L Number	Hits	Search Text	DB	Time stamp
1	2	(distinct adj population) same particle same (magnetic adj separator)	USPAT; US-PGPUB; EPO; DERWENT	2004/09/29 20:21
2	3	kraus.in. and (magnetic adj separator)	USPAT; US-PGPUB; EPO; DERWENT	2004/09/29 18:33
3	25	kraus.in. and (magnetic adj particle)	USPAT; US-PGPUB; EPO; DERWENT	2004/09/29 18:34
4	1166	multiple adj detection	USPAT; US-PGPUB; EPO; DERWENT	2004/09/29 18:35
5	2	(multiple adj detection) same (magnetic adj particle)	USPAT; US-PGPUB; EPO; DERWENT	2004/09/29 18:35
6	26	(multiple adj detection) and (magnetic adj particle)	USPAT; US-PGPUB; EPO; DERWENT	2004/09/29 18:38
7	2	436/526.ccls. and (multiple adj detection)	USPAT; US-PGPUB; EPO; DERWENT	2004/09/29 18:39
8	174	436/526.ccls. and population	USPAT; US-PGPUB; EPO; DERWENT	2004/09/29 19:00
9	2	do.xa. and (multiple adj3 microsphere)	USPAT; US-PGPUB; EPO; DERWENT	2004/09/29 19:06
10	2	5380663.pn.	USPAT; US-PGPUB; EPO; DERWENT	2004/09/29 19:08
11	28	(different adj2 bead) and 436/526.ccls.	USPAT; US-PGPUB; EPO; DERWENT	2004/09/29 19:14

US005932097A

United States Patent [19]

Wilson

[11] Patent Number: 5,932,097

[45] Date of Patent: Aug. 3, 1999

[54] **MICROFABRICATED MAGNETIC PARTICLES FOR APPLICATIONS TO AFFINITY BINDING**

[75] Inventor: **Robert John Wilson**, Cupertino, Calif.

[73] Assignee: **International Business Machines Corporation**, Armonk, N.Y.

[21] Appl. No.: **09/153,985**

[22] Filed: **Sep. 16, 1998**

Related U.S. Application Data

[63] Continuation of application No. 08/982,019, Dec. 1, 1997.

[51] **Int. Cl.**[6] **B01P 35/06**

[52] **U.S. Cl.** ..... **210/222**; 252/62.51 R; 427/127; 435/173.1; 435/173.9; 436/526

[58] **Field of Search** ..... 210/222, 223, 210/6 AS; 252/62.51 R; 427/127, 131; 435/173.1, 173.9; 436/526

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Primary Examiner—David A. Reifsnyder
Attorney, Agent, or Firm—Thomas R. Berthold

[57] **ABSTRACT**

Ferromagnetic or antiferromagnetic particles are used for selective separation of affinity bound partners in solution. The magnetic particles have large magnetic moments which can be made such that different strengths of magnetic moments and/or different magnetic field dependencies can be used to allow for separation of several affinity partners simultaneously.

6 Claims, 7 Drawing Sheets

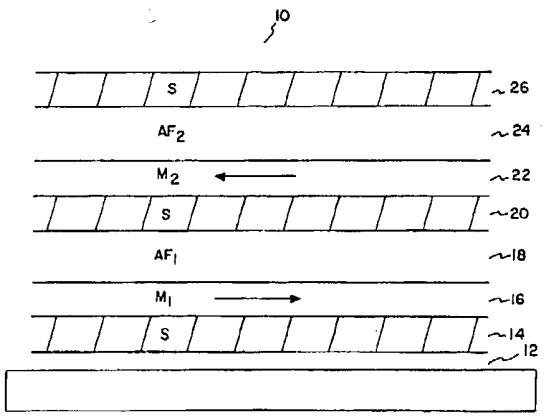

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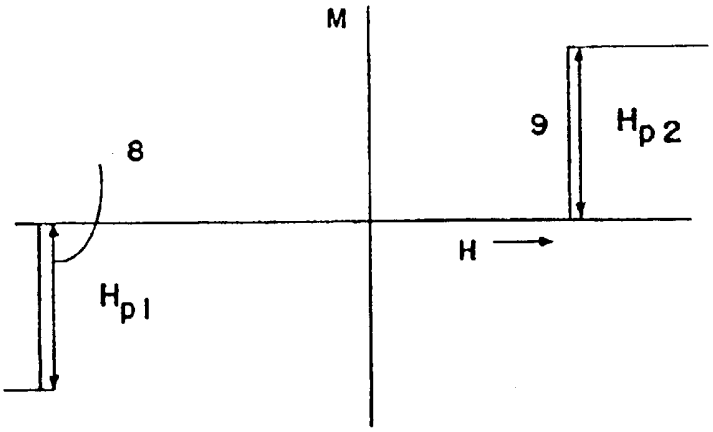
FIG. 1



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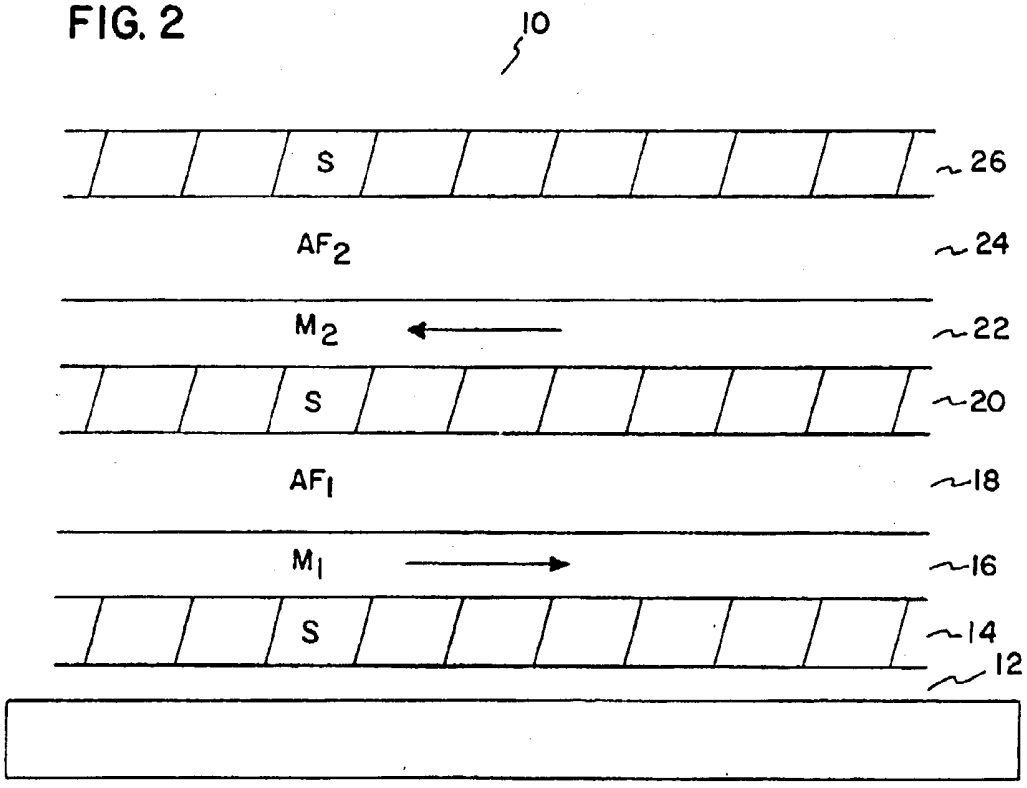

FIG. 3

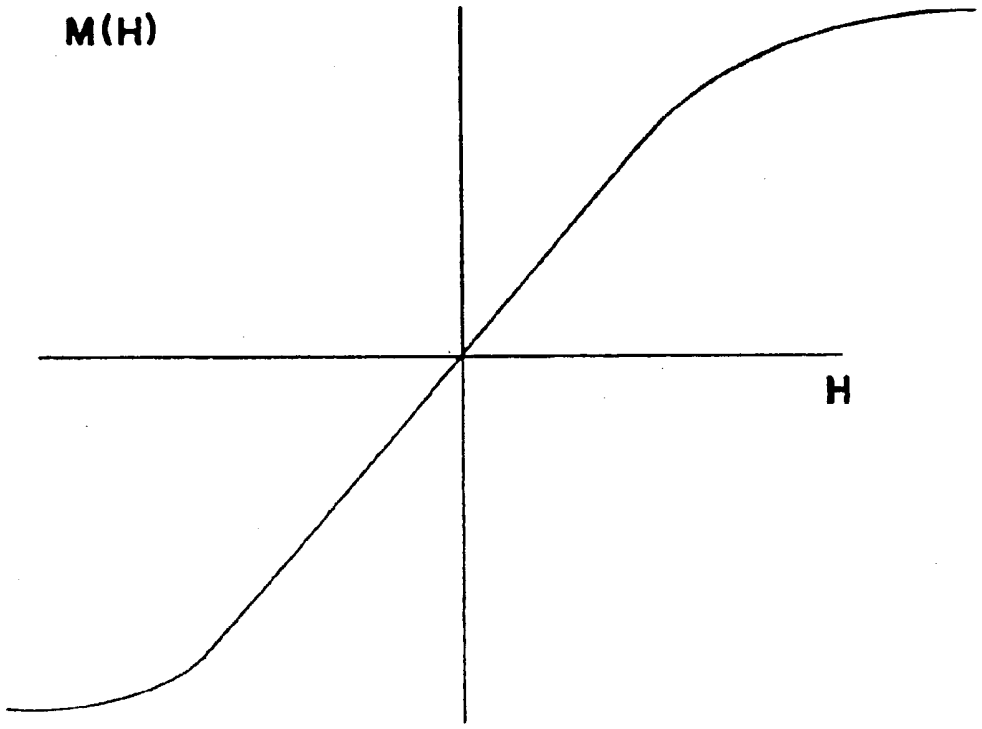

FIG. 4A

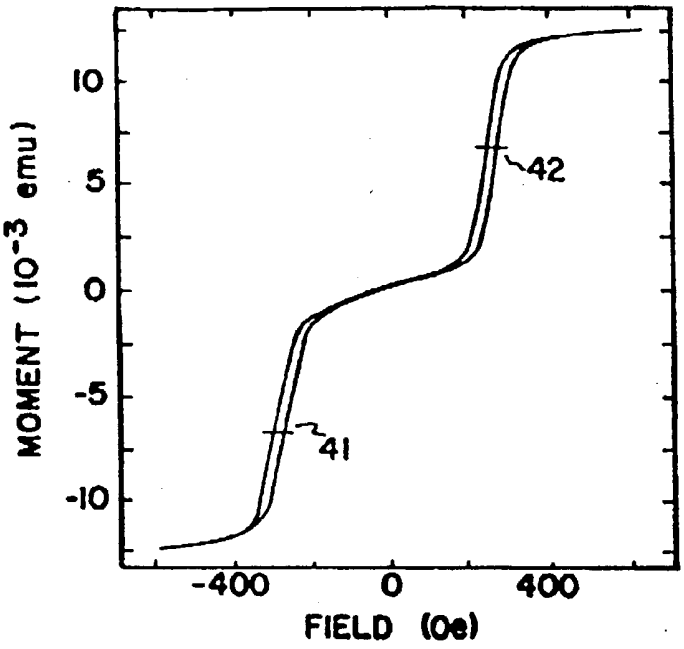
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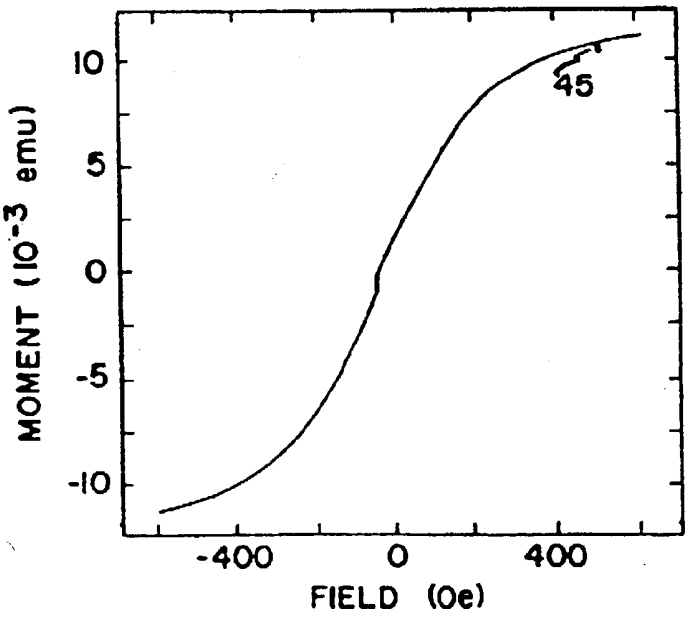
FIG. 4B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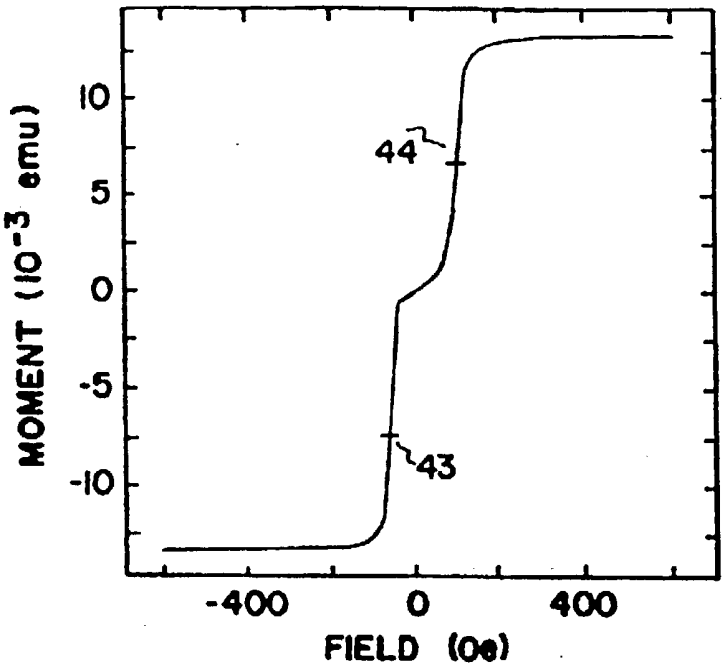
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FIG. 4D

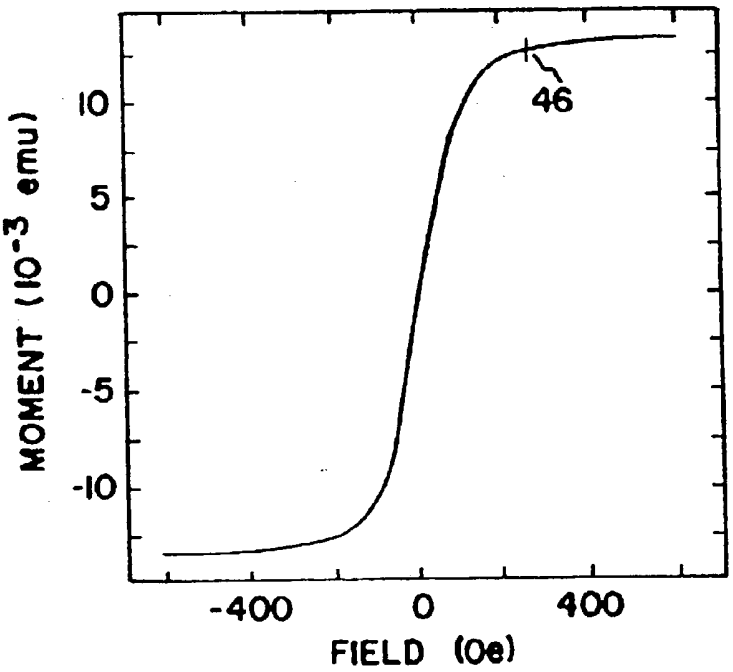
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FIG. 5A

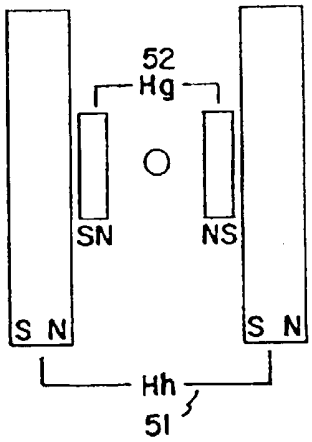


FIG. 5B

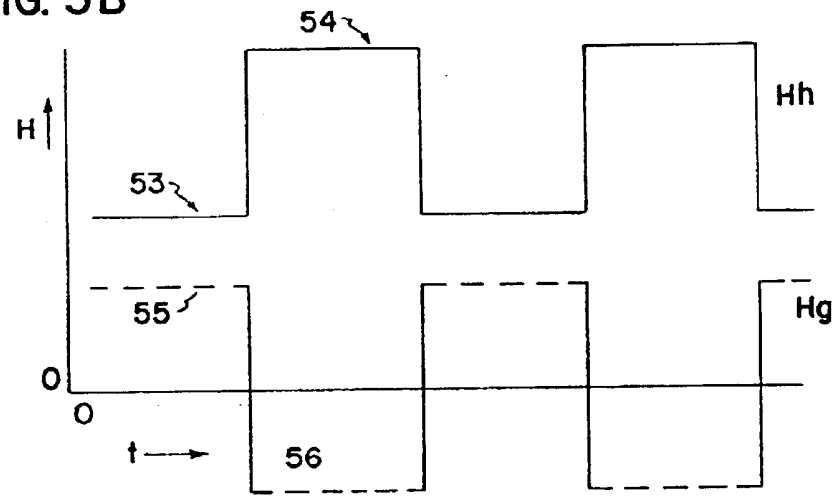


FIG. 5C

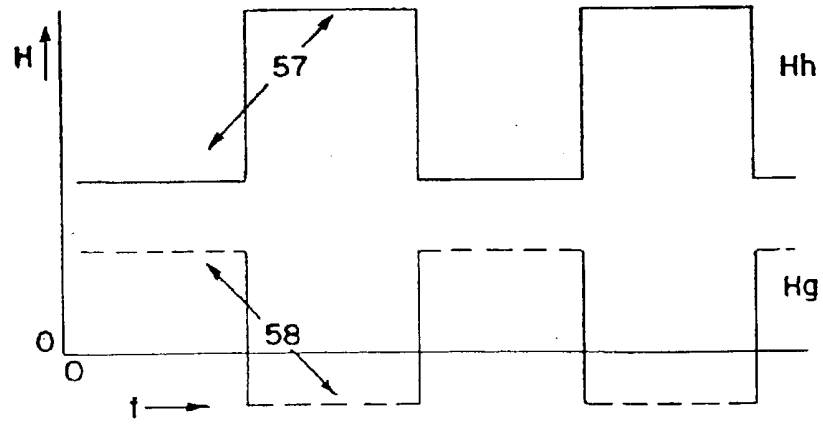
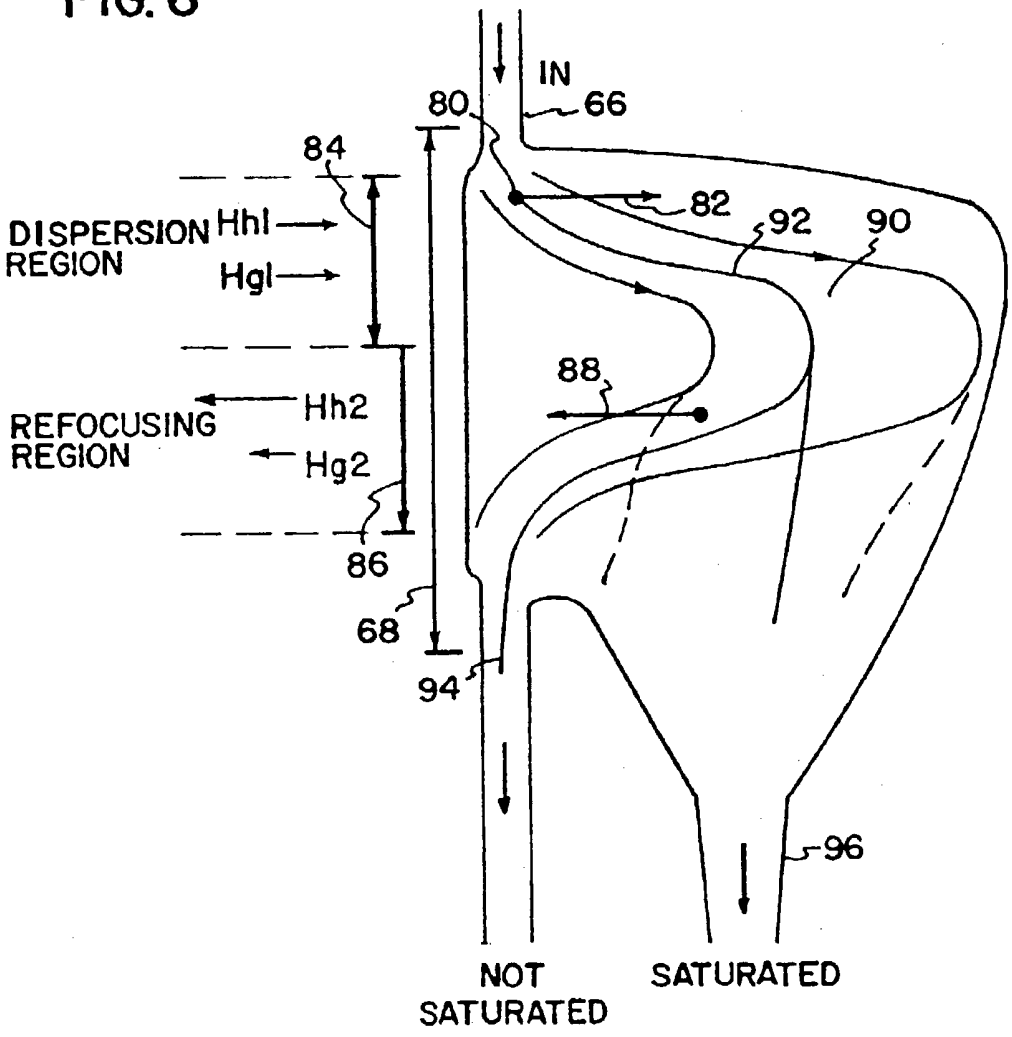


FIG. 6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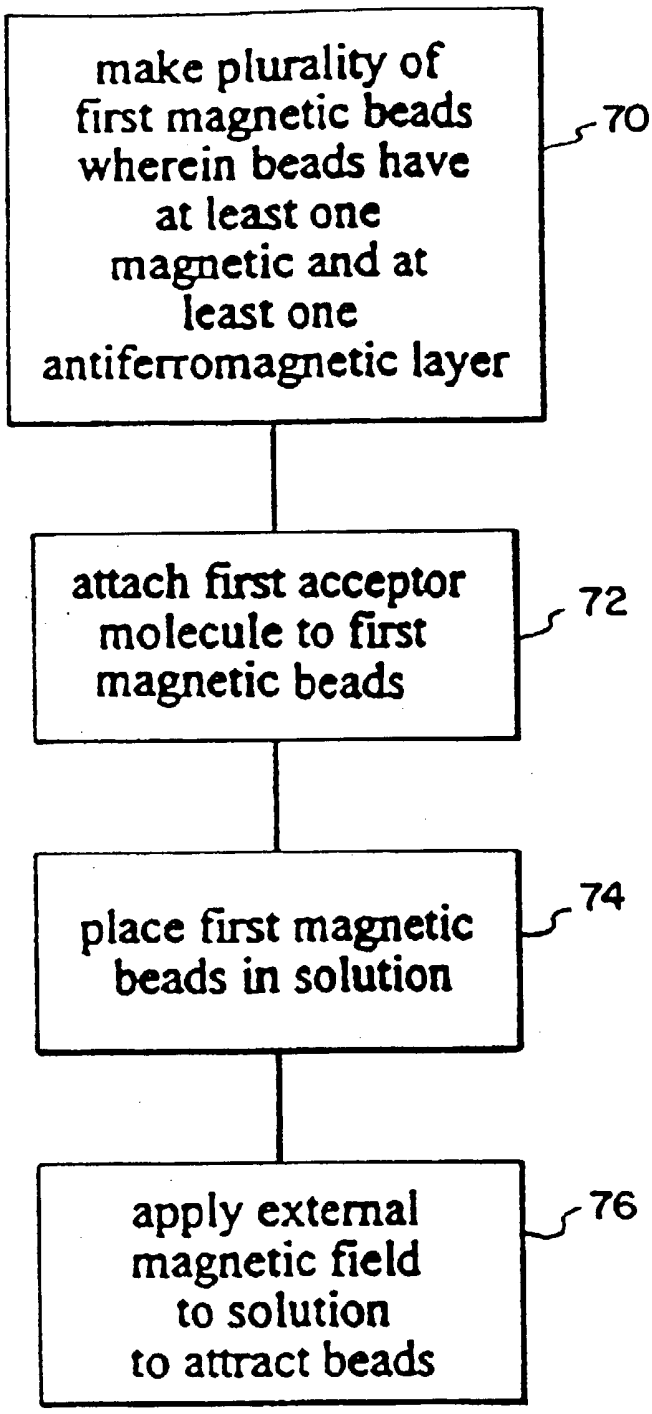
make plurality of first magnetic beads wherein beads have at least one magnetic and at least one antiferromagnetic layer
70
attach first acceptor molecule to first magnetic beads
72
place first magnetic beads in solution
74
apply external magnetic field to solution to attract beads
76

FIG. 7

MICROFABRICATED MAGNETIC PARTICLES FOR APPLICATIONS TO AFFINITY BINDING

RELATED APPLICATION

This application is a continuation of application Ser. No. 08/982,019 entitled MICROFABRICATED MAGNETIC PARTICLES FOR APPLICATIONS TO AFFINITY BINDING, filed Dec. 1, 1997, still pending.

BACKGROUND OF THE INVENTION

1. Field of the Invention

This invention relates generally to magnetic particles, and in particular to a method for fabricating magnetic particles for applications to affinity binding.

2. Description of Related Art

Magnetic particle (also known as magnetic bead) techniques have been established as valuable tools in several areas of biotechnology. The efficacy of these techniques derives from the basic property that the beads may be chemically attached or conjugated to various biochemical molecules with selective target recognition capabilities (such as proteins and nucleic acids) and added to extremely complex reaction mixtures (such as whole blood or fragmented cells in solution) so that the resulting affinity bound complexes become magnetic entities. In separations these complexes are selectively captured by a magnetic field followed by removal of unwanted impurities. Other applications, which include spatial confinement and magnetically induced aggregate formation, also exist.

The uses of magnetic bead technologies in nucleic acid scientific research include purifications for sequencing DNA, for isolating expressed RNA or proteins in differentiating cell lines, for constructing differential c-DNA libraries, for sorting chromosomes and large DNA molecules, and for isolating DNA binding proteins. There is currently a very large medical effort at whole cell separations for treatments such as bone marrow transplantation and organ transplant tolerance generation.

One limitation associated with the present technology is that magnetic separation based on only a single target is carried out at a given time, making potential multiple separations difficult, time consuming, and expensive. Further, a second separation on a given subject may not achieve optimal results, because the errors of the separations may be additive. If sufficiently distinctive magnetic particles could be produced and coupled to distinct "recognition" molecules then it might be possible to separate the particles, and their bound targets, into different groups which reflect the type and number of bound magnetic particles. One example of the advantages offered by this capability is found in the literature pertaining to bone marrow transplantation. Here it found that separations based a single cell surface marker are routinely accomplished by magnetic bead techniques while separations based on multiple cell surface markers resort to fluorescence activated cell sorters which must analyze cells on an individual basis.

It can be seen, then, that there is a need for magnetic particles that have distinct and controllable magnetic properties and that can be attached to genetic material or other objects of interest.

SUMMARY OF THE INVENTION

To overcome the limitations in the prior art described above, and to overcome other limitations that will become apparent upon reading and understanding the present specification, the present invention discloses a method and apparatus for fabricating distinctive magnetic particles for affinity binding applications. The invention is discussed in detail for the application of biological separations, but is not limited to this application. The method comprises the steps of making a plurality of first magnetic beads having a first magnetic moment, making a plurality of second magnetic beads having a second magnetic moment, attaching a first acceptor molecule to the first magnetic beads, attaching a second acceptor molecule to the second magnetic beads, placing the first magnetic beads and the second magnetic beads into a solution, and applying an external magnetic field to the solution, whereby the first and second magnetic beads are caused to move at different rates by application of a magnetic field. The velocities of the beads depend upon viscous drag forces, the applied magnetic field and field gradient, and on the magnetic moments of the first and second magnetic beads, which may also depend on the magnetic field. Various advantages and features of novelty which characterize the invention are pointed out with particularity in the claims annexed hereto and form a part hereof. However, for a better understanding of the invention, its advantages, and the objects obtained by its use, reference should be made to the drawings which form a further part hereof, and to accompanying descriptive matter, in which there is illustrated and described specific examples in accordance with the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Referring now to the drawings in which like reference numbers represent corresponding parts throughout:

FIG. **1** shows the behavior of the magnetic moment of a typical structure of the present invention in the presence of a magnetic field applied along the magnetic pinning axis, also referred to as the easy axis;

FIG. **2** shows a typical layer structure used for making the magnetic particles of the present invention;

FIG. **3** shows the magnetization curve for the case of right angle alignment between the applied field and the particle pinning axis for the present invention;

FIGS. **4A–4D** show magnetization curves for distinct particle types with the magnetic field parallel and perpendicular to their pinning axes;

FIGS. **5A–5C** illustrate one possible embodiment for separating the magnetic particles of the present invention using time dependent magnetic fields;

FIG. **6** illustrates an alternative embodiment employing spatial dependence and static fields to separate particles of the present invention; and

FIG. **7** illustrates the method steps used in practicing the present invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

In the following description of the preferred embodiment, reference is made to the accompanying drawings which form a part hereof, and in which is shown by way of illustration a specific embodiment in which the invention may be practiced. It is to be understood that other embodiments may be utilized and structural changes may be made without departing from the scope of the present invention.

Overview

The use of magnetic particles or other markers to separate desired or undesired molecules, antigens, or antibodies from

human blood or tissue is presently receiving enormous effort. The magnetic particles at one current focus of study are uniform, approximately 5 micron diameter, polystyrene spheres which are made superparamagnetic by including up to 35% (w/w) of magnetic iron oxides. Variants of the current state of the art include the use of a heterogeneous size distribution for the particles, the use of small (<0.1 micron) superparamagnetic particles, and the use of demagnetized ferromagnetic particles.

The application of magnetic bead technologies in medicine to separate intact cells uses the magnetic beads coated with monoclonal antibodies or other specific binding partners to cell surface antigens which allow cells with desirable properties to be purified from complex mixtures.

Generally such work involves cell surface protein markers, which are denoted by a Cluster of Differentiation nomenclature (e.g., CD21), that specifies the type of cells expressing each marker. Extensive commercial development and marketing of magnetic cell sorting equipment directing at monitoring, isolating and purifying various classes of lymphocytes of the immune system is presently underway.

Prominent efforts currently involve separating immune system stem cells (CD34), which primarily reside in bone marrow, and divide and differentiate to form all classes of cells involved in the immune response. The motivation behind this research is to isolate such cells from an individual or donor in order to repopulate the immune system of patients who have undergone radiation treatments or chemotherapy at dosages fatal to stem cells. Similar efforts are directed at removing cytotoxic T-cells from allogeneic (distinct genomes) bone marrow donors.

In some of these applications it is desired to separate cells on the basis of the presence (or absence) of more than one distinct cell surface marker. Such separations presently employ fluorescence activated cell sorting, on a cell by cell basis, and are used in clinical applications. Unfortunately, the current state of the art is limited to a few thousand cells per second rate, which requires about a day of instrument and operator time to sort enough cells (typical rescue doses are about 2×10^6 cells) that are about 1% abundant after a brief pre-purification using cell density dependent centrifugation.

Current magnetic bead techniques do not require the examination of each individual cell and thus require less sophisticated equipment and less operator attention as the separation is accomplished by simply placing the solution containing the magnetic bead-cell complexes in a magnetic field gradient for times of about an hour.

Prior Art Compared to the Present Invention

One limiting feature of prior magnetic bead techniques is that only one cell marker is targeted at a given time. The present invention uses magnetic particles with sufficiently distinctive character such that several such separations are performed in parallel or that fractions which are bound to particular combinations of markers can be isolated. The present invention achieves this goal by constructing particles whose magnetic moments are controlled both in magnitude and in magnetic field dependence using techniques developed for magnetic domain stabilization in sensors for magnetic storage applications. A key feature of the present invention is that multiple layers of ferromagnetic materials, whose properties are modified by the use of antiferromagnetic coupling or antiferromagnetic exchange bias, are used to tailor the magnetic response of particles for multiple separations or other applications. This feature is absent in prior art.

Basic Physical Considerations

Affinity bound magnetic beads in solution experience forces associated with magnetic fields, gravity, and collisions and electrical interactions with other solution components and container walls. Wall interactions are generally well understood and here the solution environment is considered only in terms of diffusion and viscous drag forces. Electrical forces in ionic aqueous media are generally of short range and, although useful in the context of colloidal stability and wall interactions, are well known in the art. The magnetic forces of interest in the present application arise from the applied magnetic field, and from the magnetic fields of the magnetic beads themselves. The latter forces are important because they can lead to the formation of aggregates of magnetic particles and their complexes.

A single separation is performed by applying a magnetic force that is not overwhelmed by gravitational forces. The application of such a magnetic force draws the magnetic beads into a region determined by the magnetic field so that non-magnetic components can be eliminated. If the bead-target complexes and the magnetic forces are all identical, and gravitation, diffusion and laminar flow issues are manageable, then the complexes all move at the same rate along a separatory column. However, in a single separation the goal is merely to collect all beads and complexes, and thus, there is no need for a column, homogenous beads or magnetic forces, and little concern regarding aggregation forces provided they are sufficiently weak to allow the beads to be suspended for mixing and binding or unbinding of their targets.

To perform a simultaneous separation of multiple targets, two magnetically distinct types of beads are employed by placing a mixture of the bound beads at one location in a generalized separatory column. In this case, the magnetic forces will be different for the different types of beads. If the bead-target complexes are all identical with respect to viscous forces and diffusion, then the two types of bead-target complexes move at different rates in the applied magnetic field and physically separate into two bands. It is clear that in the case of a multiple separation, aggregation of the distinct types of beads will destroy the perfection of the separation. Also, aggregation of beads of the same type may modify the magnetic and viscous drag forces on the aggregate and therefore alter the velocity of the affected complexes, resulting in a deviation from the ideal of separation into two bands.

The consequences of aggregation in the case of a multiple separation depend on the desired yield and purity of the separation. The yield and purity are limited by the loss of complexes from the desired bands and by the appearance of inappropriate complexes within the desired bands, respectively.

Finally, a separation can be performed wherein entities which carry multiple binding sites for each of two or more distinct markers simultaneously appear on a single target. The magnetic forces then depend on both the number and type of magnetic beads in the complex so that, even for identical viscous drag effects, multiple bands are expected. As a further complication, allow that the target-bead complexes can have significant variations in size and shape so that significant variations in viscous drag must be accommodated. Now the whole notion of separation into bands becomes questionable. This is the regime where separations resort to fluorescence activated cell sorting. This technique uses distinct fluorescently labeled beads, again conjugated to specific recognition molecules, so that the type and number

of attached beads are distinguished on the basis of their optical fluorescence spectrum and intensity, respectively. This technique is not subject to strong complications resulting from variations in viscous drag coefficients, but does require that measurement and separation be made on a cell by cell basis.

The prospects for the extension of magnetic bead techniques to this realm would be markedly improved if beads with sufficiently distinctive magnetic characteristics and methods for overcoming possible problems associated with variations in viscous drag coefficients were made available. Consider first the distinctive magnetic characteristics aspect.

The magnetic force, F, on a magnetic bead due to an applied magnetic field is:

$$F=-\nabla(M\times H).$$

Here H is the external magnetic field and M(H) is the magnetic moment of the bead. For superparamagnetic beads, used in prior art in fields well below the saturation field (typically many thousands of Oersteds):

$$M(H)=\chi HV$$

where χ is the superparamagnetic susceptibility and V the particle volume. In principle distinct types of superparamagnetic beads could be made, for example, by altering χ through the magnetic loading or by altering V so that particles of different moments are formed. The net moment of an assembly of two types of such particles, containing n_i particles of type i, is $M=n_1M_1+n_2M_2$. In an ideal case where a column separation yielded discrete bands for each possible M, then n_1 and n_2 could be deduced for each band and indexed according to their content; i.e., fractions containing M_1 and M_2, M_1 not M_2, and M_2 not M_1. It is evident from the above that these particular fractions may occur within several bands because of variability in n_i, which is often not critical. If variations in viscous drag coefficients for the bound complexes or variations in the beads themselves cause deterioration of the desired bands, this method could fail because the velocity and location of a particular bound complex would then not provide sufficient information to determine the presence and/or absence of M_1 and/or M_2.

The present invention involves the fabrication of particles with distinct magnetic signatures so that the type of beads in a complex can be determined, independent of the value of n_i. These particles are layered structures wherein multiple ferromagnetic layers are constrained to have nearly zero net magnetic moment at zero magnetic field, to minimize aggregation problems. The magnetic moment dependence on the magnetic field is different for the distinctive particle types and is nonlinear for magnetic fields in the easily obtained range of tens to several hundreds of Oersteds. The distinctive effective susceptibilities allow an independent determination of the types of beads in the complex, much as distinctive spectral characteristics are exploited in the case of fluorescent beads. Furthermore, the nonlinear magnetic characteristics permit the use of separation schemes which facilitate the reduction of problems associated with viscous drag variability.

Forces Required for Separation

There are several physical issues and constraints that need to be examined before proceeding to understand the structures that are proposed. First consider colloidal stability, referring to the tendency of magnetic particles to settle out of solution. A rough estimate of stability can be made by comparing the gravitational potential energy difference for a particle over a cm height range with the thermal energy kT where $k=1.38\times10^{-16}$ ergs/K is the Boltzmann constant and T is the temperature. For a sphere of radius r and density ρ in a liquid of density ρ_0, the size is limited to:

$$r=0.02\ \text{micron}\times(\delta\rho)^{-1/3}$$

where $\delta\rho=\rho-\rho_0$ is in gm/cm^3. For the materials used in the formation of the magnetic structures herein, $\delta\rho=10$, settling will occur unless very small particles or low density coatings are employed. However, mild agitation is sufficient to overcome simple settling effects.

A more stringent criteria is that the magnetic forces be comparable to gravitational forces so that gravitational settling does not overwhelm magnetic separation. Equating the magnetic and gravitational forces on a particle gives:

$$MV\nabla H=\delta\rho Vg,$$

where M is the magnetization, V is the particle volume, ∇H is the gradient of the magnetic field and g=1000 cm/sec^2. For ferromagnetic materials M is about 10^3 emu/cm^3 and $\rho=10$ gm/cm^3, so that $\nabla H=10$ Oersteds/cm. This magnetic field gradient is readily obtained. This calculation assumes the particles are comprised solely of ferromagnetic materials, but other materials can clearly be included within the beads if the field gradients are appropriately modified.

Particle Motion

The magnetic forces should also be sufficiently strong that the induced motion is not dwarfed by diffusion processes. The difflusion constant D is given by the Einstein-Smoluchowski equation:

$$D=kT/f$$

where f is the frictional drag coefficient.

For a small particle of radius r and small velocity v in an aqueous medium of viscosity $\eta=10^{-2}$, the Reynolds number is small so that Stokes' equation applies and f is only weakly shape dependent. For an r=1 micron radius sphere $f=6\pi\eta 2r=4\times10^{-5}$; so D is about 10^{-9} cm/sec^2.

Since $\Delta x=(2Dt)^{1/2}=0.6$ microns ($t^{1/2}$), diffusion is a slow process for such particles.

Stokes' equation also gives a particle translational velocity for a force F as v=F/f. The gravitational force on a 1 micron radius particle of $\rho=10$ is 4×10^{-8} dynes, so $v=10^{-3}$ cm/sec. This velocity is reasonably small to allow time for separations.

Magnetic Aggregation at Zero Field

Concerns with aggregation of particles have already been expressed in several contexts. To understand the origin of these concerns, compare the attractive dipole-dipole interactions with those of thermal agitation. For single domain ferromagnetic particles with magnetizations of M=1000 emu/cm^3 and radius r, one equates the magnetic dipole-dipole interaction energy at contact, $M^2/(2r)^3$ ($M=4/3\pi r^3M$) with kT. This equality is satisfied for a particle of r=0.003 micron, which would require a truly ferromagnetic magnetic particle to be of extremely small dimensions. It is for this reason that most particles employed in present context are superparamagnetic. In superparamagnetism the magnetic alignment of the internal spins fluctuates due to thermal agitation, so that the net moment in zero applied field vanishes but monotonically increases to a saturation value at a saturation field which is typically many kiloOersteds. In

the present invention, the magnetic moments of the individual ferromagnetic layers within a particle can be spatially oriented so that the net moment vanishes at zero field, but the saturation fields can be conveniently adjusted in the sub kiloOersted range.

Comparison of Superparamagnetism with Ferromagnetism

For superparamagnetic beads of prior art at fields below saturation, the magnetization $M=_{\chi}H$ with $_{\chi}=10^{-2}$. The magnetic moment of a 5 micron diameter superparamagnetic bead is then roughly $M=6\times10^{-13}H$ emu. For particles using this invention, the full saturation magnetization can be obtained at modest fields where the internal ferromagnetic layers become magnetically aligned. For a similar sized bead based on the present technology consider a 5 micron×5 micron×t(nanometer) permalloy film, where M is about 2×10^{-11} t (emu), so that a 1 nanometer (nm) thick ferromagnetic film has a moment comparable to a 5 micron diameter superparamagnetic bead in a 100 Oe field. There is thus much freedom to add additional materials to manipulate the internal ferromagnetic thin films while maintaining magnetic moments and dimensions comparable to those of prior art. Such added materials could also include polymer encapsulants and materials to aid in non-covalent or covalent attachment of biological recognition molecules. An overview of suitable methods for the attachment of biological recognition molecules is given below. The particle dimensions can also be adjusted to improve performance or reduce costs. For 5 micron particles, the number of particles available from a 1 cm^2 film is roughly $4\times10^6/cm^2$ which is comparable to the stem cell "rescue dose" for one human.

PROPERTIES AND PREPARATION OF MAGNETIC PARTICLES

The following section describes the properties and preparation techniques used in the present invention for the magnetic particle of the present invention.

Particle Manufacturing and Overcoming Aggregation

The methods used in the fabrication of certain types of magnetic heads can be directly adapted to allow the use of strongly ferromagnetic materials in structures which have no net dipole moment in zero field. These methods rely on materials and structures which produce ferromagnetic layers which align in an antiparallel fashion in zero field through the use of antiferromagnetic exchange or magnetostatic coupling of magnetic layers separated by non-magnetic spacers, or on ferromagnetic layers which are "pinned" in different directions by exchange coupling to antiferromagnetic layers. An example of the latter type of structure and coupling mechanism are implicit in the spin valve. Here, one has a ferromagnetic layer which is pinned along a pinning axis by exchange coupling to an antiferromagnetic layer.

There is also a "free" ferromagnetic layer which is magnetically isolated from the pinned layer by a nonmagnetic spacer. Each ferromagnetic layer has a magnetic moment which displays a hysteretic M(H) loop as the field is varied. The free layer loop is centered at low fields while the pinned layer loop center can be offset to field values ranging up to thousands of Oersteds through the use of exchange bias. Since the magnetic moments of the two layers "flip" at different fields, the moments of the layers can be aligned along different directions, depending on the value of the applied field. For the present invention, it is desirable to limit magnetic aggregation by assuring that the net moment be small in zero field. To achieve this goal, two layers of equal moment can be pinned in opposite directions so that there is an adjustable range where magnetic fields along the easy axis leave the moments antiparallel. FIG. **1** shows the magnetic moment as a function of an external field applied along the pinning axis for a pair of ferromagnetic films with equal magnetic moment and opposite pinning fields $H_{\rho1}$ **8** and $H_{\rho2}$ **9**. Fields between these values produce no net moment, but fields outside of this range induce the pinned layers to take on parallel alignment. Other forms of magnetic coupling which similarly control the relative alignment of layered magnetic films can be similarly exploited.

FIG. **2** shows a typical layer structure **10** used for making the magnetic particles of the present invention. A substrate with a lift off layer **12** is used as the base for growing or sputtering additional layers onto the overall structure **10**. Spacer layer **14** is applied to lift off layer **12**, either via sputtering, vacuum deposition, or other attachment process. The spacer layer **14** may or may not be necessary to establish the proper magnetic properties or to aid in the binding of a coating to the structure. A first magnetic layer **16** is then coupled to the spacer layer **14**. The thickness of magnetic layer **16** depends on the dipole moment and pinning field desired, on the materials being deposited, and the overall moment of the structure **10**. Typical magnetic layer **16** thicknesses are between 1 and 50 nanometers.

A first antiferromagnetic layer **18** may then coupled to first magnetic layer **16**. The antiferromagnetic layer **18** biases the dipole moment of magnetic layer **16** to prefer orientation along a given direction, called pinning the dipole moment of magnetic layer **16**. Typical antiferromagnetic layer **18** thicknesses are between 0 and 50 nanometers. Typical antiferromagnets used for exchange biasing include certain oxides of iron and nickel and several manganese alloys such as FeMn, NiMn, IrMn, PtMn, etc.

A spacer layer **20** may then be attached to antiferromagnetic layer **18**. The spacer layer **20** provides separation between the antiferromagnetic layer **18** and further layers of magnetic material, and may be of value for helping to define the magnetic characteristics of subsequent layers within the structure **10**. This spacer layer may be unnecessary. For example, if spontaneous antiferromagnetic coupling is used, then the antiferromagnetic layer **18** may be omitted and the spacer layer **20** thickness and composition adjusted to obtain the desired antiferromagnetic coupling.

The structure **10** then has a second magnetic layer **22** coupled to spacer layer **20** to allow control of the net magnetic moment of the ferromagnetic layers which comprise the structures **10**. To alleviate the problem of aggregation, the orientation of the dipole moment of second magnetic layer **22** is typically substantially opposite to that of first magnetic layer **16**, but can be at other angles, including parallel to the dipole moment of first magnetic layer **16**, depending on the strength of the dipole moment and overall characteristics desired for the structure **10**.

A second antiferromagnetic layer **24** may then be coupled to second magnetic layer **22**. The second antiferromagnetic layer **24** keeps the dipole moment of magnetic layer **22** oriented in a given direction, called pinning the dipole moment of magnetic layer **22**. This second antiferromagnetic layer may be unnecessary if spontaneous antiferromagnetic coupling is obtained through control of spacer layer **20** or from demagnetization fields.

A spacer layer **26** is then attached to second antiferromagnetic layer **24**. The spacer layer **26** may or may not be

necessary to protect the ferromagnetic and antiferromagnetic layers and may aid in the binding of a coating to the structure.

The relative pinning directions of the ferromagnetic layers **16** and **22** can be different and can be set by several methods. For some antiferromagnetic exchange bias materials, the ferromagnetic layers can be set in opposite orientations during manufacture of the structure **10** by reversing an applied magnetic bias field when the second magnetic layer **22** is grown. The layers can also be set antiparallel after the structure has been grown by using antiferromagnets with different elevated blocking temperatures for layers **18** and **24**. In this case the pinning of each ferromagnetic layer is set by the field which is present when the sample is cooled through the blocking temperature of its biasing antiferromagnet.

The structure **10** shown in FIG. **2** is shown for illustration only and is not meant to limit the scope of the invention. Other structures, using more or less spacer layers, more or less magnetic layers with varying magnetic strength, varying directions of dipole moments, and varying numbers of antiferromagnetic layers are possible within the scope of the present invention.

Fabrication of structures **10** are straightforward using sputtering, vacuum deposition, growth, vapor deposition, and epitaxial growth procedures. The structure **10** can be grown as a film and then separated into particles using a number of techniques, including using photoresist and subtractive etch methods with a suitable underlayer or lift off layer **12** to allow their release from the substrate. Alternatively, a suitably injection molded or photoresist fabricated patterned template and a release agent could allow simple lift off from a reusable template. Deposition onto performed particles is still another alternative.

The value of the pinning fields can be easily estimated. As an example, for an 80:20 NiFe layer (shown as layers **16** and **22** in FIG. **1**) of 5 nanometer thickness pinned by 50:50 MnFe of 10 nanometer thickness (shown as layers **18** and **24** in FIG. **1**), the value of H_ρ (in Ta/NiFe/MnFe/Ta layer structures) is about 300 Oe and scales as the inverse of the magnetic layer thickness. Hence the same moment can be obtained by using two exchange biased 2.5 nanometer NiFe films but the pinning field for each would increase to about 600 Oe. It is well known that the magnetic moments of films may not be linear in the film thickness for films in this thickness range but adjustments in the physical thickness to compensate for such deviations are straightforward. Similarly, it is well known that the value of the exchange coupling depends upon the structure of the deposited material but this can be determined and controlled empirically. By laminating different numbers of such layers (**16** and **22**) with different ferromagnetic layer **16** and **22** thicknesses both the saturation moments and fields where the ferromagnetic saturation occurs can be varied. If several groups of distinct particles are made and each group of structures is conjugated to distinct biological recognition molecules, the present invention has the ability to sort the different biological marker bearers by using the field dependence of the mobility of each magnetic particle group.

Calculation of Energy of a Particle in an Applied Field

The idealized magnetic behavior shown in FIG. **1** appears to offer particles whose magnetic moment abruptly switches on when the applied field increases through the pinning field. The simple discussion above is qualitatively correct for the case where the applied field is along the pinning axis. However, if the particles are free in solution they can easily rotate their pinning axes away from the applied field direction in order to attain the minimum energy orientation. This rotation arises because of the torque, M×H. For a small particle of radius r, this torque corresponds to a rotational force F_{eff}=MH/r which is large compared to the forces due to typical magnetic field gradients. If H/X represents the magnetic field gradient, then F_{eff} is enhanced over the gradient induced forces by the ratio X/r which is 1000 for x=1 mm and r=1 micron. A similar argument applies if a particle is initially aligned with the thin magnetic film dimension perpendicular to the applied magnetic field direction, but now with r replaced by t in the nanometer range. Hence, very strong torques operate to align particles so that the applied filed is in the plane of the ferromagnetic films. Thus the orientation of the particles is strongly determined by the requirement for zero torque which occurs at the minimum energy configuration. The energy of two magnetically decoupled, magnetically soft, and oppositely pinned moments M1,2 in an applied field H_a oriented at an angle θ with respect to the pinning axis can be modeled as:

$$E=-M\{(H_{\rho 1}^2+H_a^2+2H_{\rho 1}H_a\cos\theta)^{1/2}+(H_{\rho 2}^2+H_a^2-2H_{\rho 2}H_a\cos\theta)^{1/2}\}$$

The correct branch of the square root has been chosen for $H_a<H_{\rho 1,2}$ with $H_{\rho 1,2}$ the pinning fields. When $H_{\rho 1}=H_{\rho 2}$, dE/dθ when sin (θ) cos (θ)=0. At sin (θ)=0, the particles align with their pinning axes along the applied field and the increase in the energy for one moment exactly compensates the decrease for the other. The total energy is independent of the applied field at this point of maximum energy. When cos (θ)=0, the pinning axis is perpendicular to the applied field. In this case, both moments rotate as the field is applied to have a component along the applied field of $Ha/(H_a^2+H_\rho^2)^{1/2}$. The net magnetization increases, initially linearly, and goes smoothly from 0 to 2 M, gradually saturating at fields $H_a>H_\rho$. The abrupt transition from antiparallel to parallel alignment at Hp is clearly lost. The particle itself can maintain the same rotational orientation with its pinning axis perpendicular to the field as the field is increased because the net moment produces no torque. FIG. **3** shows the magnetization curve for the case of right angle alignment of the fields of the particle of the present invention.

In a more general case where $H_{\rho 1}>H_{\rho 2}$, it is easy to show that as the field is increased the net moment first arises at right angles to the pinning direction but, as M_1 is less susceptible to rotation, the particle gradually rotates to align at an angle where the moment is between the pinning axis and its perpendicular. It is easy to show that in this case: cos $(\theta)=(H_a/2)(1/H_{\rho 2}-1/H_{\rho 1})$ for $H_a<H_{\rho 1}$, $H_{\rho 2}$. In the extreme case where $H_{\rho 1}>>H_{\rho 2}$, this expression shows that the particle rotates from the right angle orientation, preferred at low fields, to align the net moment along the pinning axis at large applied fields. Thus the saturation magnetic moment is obtained more rapidly with increasing field than in the case where $H_{\rho 1}=H_{\rho 2}$. The above model is not intended to be fully quantitative, as it neglects coercivities, anistropies, demagnetizing fields, etc.

Qualitatively similar behavior can be expected if other forms of antiferromagnetic coupling are employed. These might include antiferromagnetic or biquadratic coupling through nonmagnetic spacers or antiferromagnetic magnetostatic coupling associated with "magnetic charges" on the surfaces of patterned structures.

Constraints on Particle Manufacturing

The thickness and lamination of the magnetic layers is subject to some constraints because the self generated mag-

netic fields of the particles affect both the behavior of the magnetization and particle aggregation. These fields result from equivalent magnetic "surface charges" which occur at edges where the magnetization is normal to a surface. Demagnetization fields from a film act on the film itself to cause anisotropies and act between adjacent films to tend to align the layers in an antiparallel sense. These demagnetization fields also provide a rough estimate of the fields when 2 particles aggregate in a head to tail chain, or a side by side, antiparallel sense.

In the specific case of antiferromagnetic exchange bias, these fields can be held to small values so that they can be neglected as compared to pinning fields and the applied fields used to control the particles. The demagnetizing field can be estimated from the induced "magnetic surface charges" at the ends of a film. For length 1, width w and thickness t, the line charge density along the width is M1t. The field at the center is then roughly $Mwt/1^2$. A better estimate for a ferromagnetic NiFe film with t<w<1 and M directed along w is:

$$Hd=\pi^2 M\times(t/1)=10\times t(nm)/1(um)\ Oe.$$

The aspect ratio t/1 can be constrained to keep Hd below some tens of Oersteds which can further be kept small compared to Hp. Other shapes, such as discs, can also be used to alter the anistropy and the magnitude and spatial structure of demagnetization fields.

It is important to realize that particles containing laminated ferromagnetic layers with antiparallel moments do produce external magnetic fields. For parallel moment alignment the net moment is 2Mwt1 which acts as a dipole source from which external fields can be readily. estimated. The external fields are much weaker in the antiparallel orientation because there is no net moment. In this state, the "magnetic charges" at a given end of two antiparallel layers will have opposite signs. For antiparallel layers spaced by s, there are thus oppositely directed dipoles at each end of the particle. These dipoles have moments which are roughly Mwts=10–15 wts with w in microns and t and s in nm, which are reduced in magnitude, relative to parallel alignment, by a large value of $1/s=10^3$. External fields at large distances further become quadrupolar in nature as the end dipoles are oppositely oriented.

The possibilities of spontaneous aggregation of the particles must also be addressed. One criterion is the ratio of the attractive interaction energy and the thermal energy kT. For a single magnetic layer of dimensions 5 micron×5 micron×5 nm and magnetization of 1000 emu, the field H where MH=kT is $H=4\times10^{-4}$ Oe. Given that demagnetizing fields were estimated as below tens of Oersteds, aggregation is expected for particles whose ferromagnetic layers are aligned by a magnetic field. In the antiparallel orientation the external fields are reduced by three orders of magnitude and thermal energies are not so different from interparticle interaction energies. Further reductions in external fields in the antiparallel state can be obtained by stacking multiple magnetic layers to eliminate dipoles for quadrupoles, etc. However, we find that aggregation in the demagnetized state is not a problem as particles which have gravitationally settled into dense assemblies are easily resuspended as isolated particles by mild agitation in low field.

Aggregation problems are important in the parallel aligned state because the particles may ultimately clump together magnetically, possibly linking distinct species which one may desire to separate. Assume that all phases of particle-recognition molecule attachment and complex formation with targeted entities are performed and nonaggregated complexes are placed in a separatory apparatus and the magnetic field is subsequently turned on. To estimate the time that elapses before interparticle magnetic forces draw the particles together, consider the solution velocity as given by Stokes' equation or an aqueous solution using dipolar interparticle magnetic forces for two particles with magnetic moment M. Very roughly one finds:

$$6\pi\eta a\ dR/dt=M^2/R^4,$$

where a is the particle diameter and R the separation. A time to contact can be estimated by integration to be $t=\pi\theta a R_0^5/M^2$, where R_0 is the initial separation. For a 5 micron×5 micron×5 nm NiFe film at saturation, M is about 10^{-10} (emu). For $t=10^4$ sec, R_0 falls in the micron range so that if R_0 is held at 100 microns aggregation should not be problematic on the time scale of hours. This corresponds to particles which are suspended at reasonable densities of about $10^6/cm^3$. Actual densities can be further decreased to allow for statistical variations. This treatment assumes that the particles are interacting through a simple attractive R^{-4} force law. For magnetic dipoles the actual forces are much more complex as they are attractive for some relative orientations and positions of the dipoles and repulsive for others. It is well known that dipoles can coalesce to form chains wherein particles with parallel dipoles link together along their mutual dipolar axis via attractive forces between their respective north and south poles. If, however, parallel particles positioned in a head to tail chain configuration are rotated by 90 degrees, by rotating the external field, prior to coalescence then the forces become repulsive as like poles become proximal. Thus magnetic field rotations and or temporal reorientations may be useful for limiting aggregation phenomena.

Fabrication of Particles for Evaluation

Test structures have been fabricated using ion beam sputtering as the deposition source. First, Si wafers were cleaned and precoated with a 5 nm tantalum (Ta) film to promote adhesion of an organic lift-off layer. This layer was cast by spin coating a 1.4 micron film of precured polyimide in anisole, followed by a 30 minute bake at 100 C. Metallization was then applied to make several different examples of magnetic structures. One sample, denoted S1, had a structure:

substrate/5Ta/5Py/9FeMn/3Ta/5Py/9FeMn/3Ta/5Py/9FeMn/*revH*/3Ta/5Py/9FeMn/3Ta/5Py/9FeMn/3Ta/5Py/9FeMn/5Ta

Here the thickness of a material is given in nanometers followed by the elemental composition. Py refers to permalloy of about 80:20 Ni:Fe composition which comprised the ferromagnetic material. FeMn is of about 50:50 composition and was used as the pinning antiferromagnet. Ta spacers were used to separate magnetic materials, to set up crystalline texture advantageous for exchange bias, and as a protective cap. The notation *revH* refers to the fact that a magnetic field of some tens of Oersteds was applied to set the pinning direction throughout growth and was reversed in direction after the first set of magnetic layers was completed. The magnetization loops of this sample were recorded using vibrating sample magnetometry with the field parallel and perpendicular to the pinning axis are shown in FIGS. 4(A) and (B), respectively. A second structure, denoted S3, was grown with the following structure:

substrate/3Ta/20Cu/5Ta/15Py/9FeMn/*revH*/3Ta/15Py/9FeMn/3Ta/20Cu/5Ta.

The total Py thickness is same for S3 as for S1, but the FeMn exchange coupling is expected to be weaker for S3 because

of the thicker individual Py layers. The Cu layers in S3 were used to maintain roughly the same thickness as S1 but to also provide a distinct color. FIGS. 4(C) and (D) show the corresponding magnetic behavior for S3. The attainment of different pinning, 41—44, and saturation, 45 and 46, fields and of low magnetization in zero field are clearly evident. There are small deviations in the values of the predicted pinning fields and in the magnetic moments but these can be easily corrected by empirical modification of film thicknesses.

The wafers were then spin coated with a 1.3 micron chemically amplified polyimide resist and baked at 95 C for 5 min. They were next placed in contact with a 5 micron checkerboard mask, 1 cm^2 in area, and exposed for about 1 sec to 50 millijoules of UV radiation. A post exposure bake at 95 C for 2 min was performed and the pattern was developed using immersion in an alkaline developer for about 30 sec. The photoresist pattern was transferred into the metallization by using ion milling to remove the metal layer in regions not covered by the developed photoresist pattern. The lift off underlayer was dissolved in warm n-methyl pyrrilidinone using agitation and the bare substrate was removed from the solvent. The particle bearing solvent was transferred to test tubes and a small, 500 Oe, magnet was used to collect the magnetic particles so that the organic solvent could be extracted and exchanged for water. The distinctive particles were ultimately placed in different tubes, each containing about 1 cc of water, at an estimated density of $4\times10^6/cm^3$.

Separation of Particles in Solution

With this insight into the magnetic properties of the desired structure disclosed in the present specification, it is appropriate to discuss one mechanism of physical separation which is unique to such particles. For the present discussion, we consider the magnetic field to be comprised of two different horizontal components which are illustrated in FIG. 5(A). The first field is a relatively spatially homogenous field Hh, 51, which can be pulsed in time. The second field Hg, 52, has a relatively large gradient but a small value in the region of interest and can also be temporally pulsed. For the purpose of illustration, consider that the particles fall vertically in solution under the influence of gravity in the absence of magnetic fields. The homogenous field, Hh, is used to induce a magnetic moment in the suspended particles while the gradient field, Hg, is used to induce motion of the magnetized particles. If the gradient field is symmetrically pulsed in alternating opposite directions while Hh is held constant, then the lateral direction of motion of the magnetic particles will also alternate so that no net lateral displacement will be induced by the magnetic fields. If, however, Hh is varied between two different values, 53 and 54, synchronously with the alternation of Hg, 55 and 56, as shown in FIG. 5(B), then net lateral displacement will result if the magnetic moment depends on the value of Hh. Thus if Hh is held above the saturation field of one type of particle then no net lateral displacement will accrue for this type of particle. If Hh is held well below the saturation field for a second type of particle, whose moment is therefore roughly linear in Hh, lateral displacement will accrue.

If the structure of the pulses is made asymmetric then alternative separation schemes can easily be achieved. Suppose that Hh is switched varied between H1 and 2H1, 57, while Hg is synchronously switched between H2 and −H2/2, 58, as depicted in FIG. 5(C). Now particles whose moment is linear in Hh will not accrue lateral displacements as contrasted with particles which remain saturated. Indeed the latter particles will move only half as fast laterally and thus half as far horizontally during the reverse field cycle so that substantial horizontal displacements can accrue. It is clear that similar manipulations are possible if the pulsewidths for different signs of Hg, or variations in the sign of Hh are allowed. It is further evident that these separation schemes do not require precise linearity or total saturation as adjustments in various parameters can be made to compensate for the actual magnetic characteristics. Finally, it is also straightforward to reorient the field directions relative to gravity or to include other forces in these separation schemes to achieve systems where, for example, some species are allowed to settle while others are levitated.

FIG. 6 illustrates one possible embodiment for separating the magnetic particles of the present invention using spatially separated static fields.

Inlet 66 allows a given solution, possibly magnetic bead complexes with human blood, to enter into a region 68 that is exposed to magnetic field influences. For illustrative purposes only, the magnetic field is separated into homogenous and gradient components, Hh and Hg, and a single particle 80 is shown initially in region 68.

The magnetic moment of a given magnetic particle 80 will induce a velocity of magnetic particle 80 in direction 82 in a dispersion region 84. Similarly, refocusing region 86 will deflect particle 80 in direction 88 within refocusing region 86. Particles of various moments can be deflected different lateral distances away from the vertical trajectories associated with gravitational settling from inlet 66.

As particle 80 moves through dispersion region 84, particle 80 experiences a horizontal field Hh1 and a gradient field Hg1. Dispersion region 84 deflects particle 80 in direction 82 proportional to the strength of the magnetic moment of particle 80 and the strength of the magnetic field Hg that is applied. The vertical force is constant, and the trajectory 90 of particle 80 may have a curvature 92 associated with non-uniformities in the overall magnetic field. If variations in cell size, density, viscous drag forces, and number of particles 80 bound are present the trajectories 90 for different particles 80 will disperse.

In refocusing region 86, the magnetic field Hh2 or the gradient field Hg2 are reversed. The trajectory 90 can refocus particle 80, for example if Hh1=Hh2 and Hg1=−Hg2, because diffusion and interparticle interactions are negligible. This refocusing action will not work properly if the homogeneous magnetic field is different in the dispersing and focusing regions and the beads are designed to have a non-linear dependence of M on Hh.

For example, if Hh2=2 Hh1 and Hg2=−½ Hg1, refocusing will occur provided the magnetic moment of particle 80 is linear in H below the value 2Hh1. If this is the case, particle 80 will enter outlet 94, because the field has drawn particle 80 in direction 88 the same amount that particle 80 was dispersed in direction 82.

If M is nearly saturated at Hh1 then the refocusing deflection in direction 88 is only half of the dispersing deflection in direction 82. Thus, the saturated particles 80, which are now "focused cells," can be collected as those with saturation at fields below 2Hh1 in outlet 96. The particles from outlet 94, which did not saturate for fields below 2Hh1 can be further tested for saturation at higher fields by using the outlet 94 as an inlet to another set of fields similar to the ones described above, with different values for the field strengths. Alternative schemes are obviously possible. For example, one could choose Hh2=Hh½ and Hg2=−Hg1 to collect particles which are saturated at field values above Hh½ at outlet 94.

It is straightforward to devise schemes wherein separations for given types of markers can be obtained by connecting different sorters as serial logical elements to discriminate for or against single bead types or combinations of bead types. For example, consider a case where the presence of markers M1 and M2 are desired, while M3 is to be selected against. Presume distinct beads are available which saturate at 50, 150, and 450 Oe. First, nonmagnetic species can be trivially eliminated. To eliminate M3 containing complexes, the M3 bead can be chosen to be the 450 Oe bead and one can use Hh1=900 and Hh2=450 and Hg2=−Hg1 to reject any complex containing M3 but to refocus complexes containing M1 and/or M2 into outlet **94**. To require M2, choose M2 to correspond to the 150 Oe bead, use Hh1=50 and Hh2=100 with Hg2=Hg½. Now complexes containing only M2 will refocus at outlet **94** of the second sorter and can be discarded. Beads containing only M1 or M1 and M2 can be collected at outlet **96** and further separated by using Hi1=25 and Hh2=50 with Hg2=Hg½. Refocused beads at outlet **94** of this stage contain only M1 so that the desired M1 and M2 fraction appears at outlet **96** of this stage. The refocusing action exploited here for positive or negative selection is explicitly mentioned because the fractions which appear at outlets **94** are deliberately intended to return to their initial lateral positions independent of variations in the value of viscous drag coefficients.

This distinct separation of particles cannot be obtained at convenient magnetic fields with the superparamagnetic beads currently used because the currently used particles have very high saturation fields. Composites of small, randomly oriented or demagnetized ferromagnetic particles would suffer similar problems and others related to poor control of particle shapes, saturation fields, coercivities, or irreversible magnetization.

Observations of Test Particles in Applied Magnetic Fields

The particles were easily detected, but not resolved, by the unaided eye using specularly reflected light. Noticeable settling occurred over tens of minutes but the particles were easily dispersed by shaking. The motion of the particles in the field of a 1 in diamneter, ½ in thick Alnico magnet contacting the outside wall of the tube was easily observed and the velocity was of the order of 1 cm/sec. A noticeable enhancement in reflectivity of the dispersed particles was noticed. If a weak magnetic field, measured as 1 Oe, was applied in a direction where field induced alignment of the particles with the magnetic field in the plane of the laminated films, then an enhancement in specular reflection was observed depending on the location of the light source and observer. As previously mentioned, very weak fields are sufficient to orient the particles so that the applied field is in the plane of the magnetic films. Optical microscopes were used to observe the particles using both reflected and transmitted light. Some observations were carried out in applied magnetic fields in an apparatus which used two water cooled electromagnets driven by pulsable current sources, much as illustrated by FIG. **5**(A). The first electromagnet had 2 6" diameter coils with a 4" spacing which were connected in a configuration where the fields added to give a relatively homogeneous field of about 50 Oe/Ampere. The second electromagnet was comprised of 2 2" diameter coils at a 2" spacing which were connected in an antiparallel sense so as to obtain zero net field at their center but a field gradient of about 40 Oe/cm-Amp. Qualitative optical observations confirmed that the particle velocities showed the expected distinctive saturation characteristics as a function of the homogenous field and the expected linear variations in the gradient field amplitude. Various pulse schemes were examined to confirm the reversal of particle motion with the reversal of the field gradient or with the reversal of the homogenous field. These observations qualitatively verified that the particles behaved as expected.

Formation of chain-like aggregates was also noticeable if the settled beads were shaken once or twice by hand and then subjected to strong fields for tens of minutes at the relatively high concentrations given above. These chains allowed an easy visualization of chain rotation expected during reversals of the homogenous field and of the expected variations in the sign of inter-chain forces as a function of the relative position and orientation of proximal chains. Also, the interactions of the beads and chains with the container walls suggested some straightforward methods for using the walls to advantage during separations. For example, it was simple to adjust the magnetic field and gradient so that the trajectories of certain types of beads introduced at the top of a filled container intercepted the walls if the container which was deliberately canted relative to the vertical trajectories due to gravitation. The intercepting species dramatically slowed or stopped at contact while other beads could continuously move downward parallel to the canted wall. In a pulse mode where lateral motion periodically reversed, this led to a situation in which the wall intercepting beads were free to move during the reverse motion portion of the cycle so that they were systematically delayed in their downward motion. Field rotations were also found to be effective in inducing a type of motion which might be described as "walking". Here a bead in contact with the surface in a flat orientation for a field parallel to the wall turns upright when the field is rotated 90 deg and returns to a flat orientation when the field is at 180 deg. The beads center moved by the bead lateral dimension during the process because the bead end in contact with the surface remained stationary. There was some variability in the wall interactions between particles and/or regions of the walls in our observations of wall related phenomena—but no efforts were made here to treat or clean the as received container walls.

FIG. **7** illustrates the method steps used in practicing the present invention.

Block **70** illustrates the step of making a plurality of first magnetic beads having a first magnetic moment, the first magnetic beads having at least one magnetic layer and at least one antiferromagnetic layer.

Block **72** illustrates the step of attaching a first acceptor molecule to the first magnetic beads.

Block **74** illustrates the step of placing the first magnetic beads into a solution. Block **76** illustrates the step of applying an external magnetic field to the solution, whereby the first magnetic beads are attracted to a source of the magnetic field.

ATTACHMENT AND USE OF AFFINITY RECOGNITION MOLECULES BOUND TO MAGNETIC PARTICLES

The following sections discuss the use of the above identified magnetic particles as nuclei for affinity molecules that are bound to the magnetic particles of the present invention. As indicated above, magnetic particles according to the present invention are attached to at least one affinity recognition molecule. As used herein, the term "affinity recognition molecule" refers to a molecule that recognizes

and binds another molecule by specific three-dimensional interactions that yield an affinity and specificity of binding comparable to the binding of an antibody with its corresponding antigen or an enzyme with its substrate. Typically, the binding is noncovalent, but the binding can also be covalent or become covalent during the course of the interaction. The noncovalent binding typically occurs by means of hydrophobic interactions, hydrogen bonds, or ionic bonds. The combination of the affinity recognition molecule and the molecule to which it binds is referred to generically as a "specific binding pair." Either member of the specific binding pair can be designated the affinity recognition molecule; the designation is for convenience according to the use made of the interaction. One or both members of the specific binding pair can be part of a larger structure such as a virion, an intact cell, a cell membrane, or a subcellular organelle such as a mitochondrion or a chloroplast.

Examples of affinity recognition molecules in biology include antibodies, enzymes, specific binding proteins, nucleic acid molecules, and receptors. Examples of receptors include viral receptors and hormone receptors. Examples of specific binding pairs include antibody-antigen, antibodyhapten, nucleic acid molecule-complementary nucleic acid molecule, receptor-hormone, lectin-carbohydrate moiety, enzyme substrate, enzyme-inhibitor, biotin-avidin, and viruscellular receptor. One particularly important class of antigens is the Cluster of Differentiation (CD) antigens found on cells of hematopoietic origin, particularly on leukocytes, as well as on other cells. These antigens are significant in the activity and regulation of the immune system. One particularly significant CD antigen is CD34, found on stem cells. These are totipotent cells that can regenerate all of the cells of hematopoietic origin, including leukocytes, erythrocytes, and platelets.

As used herein, the term "antibody" includes both intact antibody molecules of the appropriate specificity and antibody fragments (including Fab, F(ab'), Fv, and F(ab')2 fragments), as well as chemically modified intact antibody molecules and antibody fragments such as Fv fragments, including hybrid antibodies assembled by in vitro reassociation of subunits. The term also encompasses both polyclonal and monoclonal antibodies. Also included are genetically engineered antibody molecules such as single chain antibody molecules, generally referred to as sFv. The term "antibody" also includes modified antibodies or antibodies conjugated to labels or other molecules that do not block or alter the binding capacity of the antibody.

As used herein, the terms "nucleic acid molecule," "nucleic acid segment" or "nucleic acid sequence" include both DNA and RNA unless otherwise specified, and, unless otherwise specified, include both double-stranded and single stranded nucleic acids. Also included are hybrids such as DNA-RNA hybrids. In particular, a reference to DNA includes RNA that has either the equivalent base sequence except for the substitution of uracil and RNA for thymine in DNA, or has a complementary base sequence except for the substitution of uracil for thymine, complementarity being determined according to the Watson-Crick base pairing rules. Reference to nucleic acid sequences can also include modified bases or backbones as long as the modifications do not significantly interfere either with binding of a ligand such as a protein by the nucleic acid or with Watson-Crick base pairing.

Methods for the covalent attachment of biological recognition molecules to solid phase surfaces, including the magnetic particles of the present invention, are well known in the art and can be chosen according to the functional groups available on the biological recognition molecule and the solid phase surface.

Many reactive groups on both protein and non-protein compounds are available for conjugation.

For example, organic moieties containing carboxyl groups or that can be carboxylated can be conjugated to proteins via the mixed anhydride method, the carbodiimide method, using dicyclohexylcarbodiimide, and the N hydroxysuccinimide ester method.

If the organic moiety contains amino groups or reducible nitro groups or can be substituted with such groups, conjugation can be achieved by one of several techniques. Aromatic amines can be converted to diazonium salts by the slow addition of nitrous acid and then reacted with proteins at a pH of about 9. If the organic moiety contains aliphatic amines, such groups can be conjugated to proteins by various methods, including carbodiimide, tolylene-2,4-diisocyanate, or malemide compounds, particularly the N-hydroxysuccinimide esters of malemide derivatives. An example of such a compound is 4(Nmaleimidomethyl)-cyclohexane-1-carboxylic acid. Another example is m-male imidobenzoyl-N-hydroxysuccinimide ester. Still another reagent that can be used is N-succinimidyl-3 (2-pyridyldithio) propionate. Also, bifunctional esters, such as dimethylpimelimidate, dimethyladipimidate, or dimethylsuberimidate, can be used to couple amino-group containing moieties to proteins.

Additionally, aliphatic amines can also be converted to aromatic amines by reaction with p-nitrobenzoylchloride and subsequent reduction to a p-aminobenzoylamide, which can then be coupled to proteins after diazotization.

Organic moieties containing hydroxyl groups can be cross-linked by a number of indirect procedures. For example, the conversion of an alcohol moiety to the half ester of succinic acid (hemisuccinate) introduces a carboxyl group available for conjugation. The bifunctional reagent sebacoyldichloride converts alcohol to acid chloride which, at pH 8.5, reacts readily with proteins. Hydroxyl containing organic moieties can also be conjugated through the highly reactive chlorocarbonates, prepared with an equal inolar amount of phosgene.

For organic moieties containing ketones or aldehydes, such carbonyl-containing groups can be derivatized into carboxyl groups through the formation of O-(carboxymethyl) oximes. Ketone groups can also be derivatized with p-hydrazinobenzoic acid to produce carboxyl groups that can be conjugated to the specific binding partner as described above. Organic moieties containing aldehyde groups can be directly conjugated through the formation of Schiff bases which are then stabilized by a reduction with sodium borohydride.

One particularly useful cross-linking agent for hydroxyl-containing organic moieties is a photosensitive noncleavable heterobifunctional cross-linking reagent, sulfosuccinimidyl 6-[4¢-azido-2¢-nitrophenylamino] hexanoate. Other similar reagents are described in S. S. Wong, "Chemistry of Protein Conjugation and CrossLinking," (CRC Press, Inc., Boca Raton, Fla. 1993). Other methods of cross-linking are also described in P. Tijssen, "Practice and Theory of Enzyme Immunoassays" (Elsevier, Amsterdam, 1985), pp. 221–295.

Other cross-linking reagents can be used that introduce spacers between the organic moiety and the biological recognition molecule. The length of the spacer can be chosen to preserve or enhance reactivity between the members of the specific binding pair, or, conversely, to limit the reactivity, as may be desired to enhance specificity and inhibit the existence of cross-reactivity.

Although, typically, the biological recognition molecules are covalently attached to the magnetic particles, alternatively, noncovalent attachment can be used. Methods for noncovalent attachment of biological recognition molecules to magnetic particles are well known in the art and need not be described further here.

Conjugation of biological recognition molecules to magnetic particles is described in U.S. Pat. No. 4,935,147 to Ullman et al., and in U.S. Pat. No. 5,145,784 to Cox et al., both of which are incorporated herein by this reference.

APPLICATIONS OF MAGNETIC PARTICLES ACCORDING TO THE PRESENT INVENTION

Magnetic particles according to the present invention can be used in many applications, both analytical and preparative. Among the analytical applications are specific binding assays such as immunoassays. Magnetic particles according to the present invention that have attached to them an antibody or other biological recognition molecule can be used in many types of immunoassays. Protocols for performing such immunoassays are well known in the art and need not be described in detail here. However, immunoassays are of two general types, sandwich and competitive. Typically, in sandwich immunoassays, a labeled antibody is used and what is detected is a ternary complex of an unlabeled antibody that is generally immobilized on a solid phase, the antigen, and a labeled antibody that also binds to the antigen. For performance of a sandwich immunoassay with magnetic beads according to the present invention, the unlabeled antibody is attached to the magnetic beads, and a labeled antibody is used. The labeled antibody can be labeled with an enzyme label, a fluorescent label, a chemiluminescent label, a bioluminescent label, a radioactive label, a dye label, a colloidal metal label, or any other label known to the art. Typically, in competitive immunoassays, the antigen or hapten to be assayed is mixed with antibody on a solid support and with a defined quantity of labeled analyte or analyte analogue. The unlabeled antigen or hapten in the sample competes with the labeled antigen or hapten for binding to the antibody on the solid support. Magnetic particles according to the present invention can be used as the solid support to which the antibody is bound. The magnetic particles are then separated from the mixture of the antibody, the particles, and the labeled component by application of magnetic force, and the presence or quantity of label associated with the magnetic particles is then determined in order to detect or determine the analyte. Various conventional washing steps can be incorporated into the procedure to reduce the background and improve the reproducibility of the immunoassay.

The foregoing discussion is intended to be illustrative rather than exhaustive, and many variations on these assay formats exist. Immunoassays employing magnetic particles are described in U.S. Pat. No. 4,935,147 to Ullman et al., and in U.S. Pat. No. 5,145,784. Magnetic particles according to the present invention that have a biological recognition marker on their surfaces can also be used for preparative purposes, i.e., for isolation and purification of a component that is bound specifically by the biological recognition molecule. Typically, the preparative procedure involves the following steps:

(1) contacting the magnetic particles with a mixture that contains the component in a suspension so that the component can be bound by the magnetic particles;

(2) separating the magnetic particles from the suspension by the application of magnetic force; and

(3) eluting the component from the magnetic particles to purify the component.

Typically, the component is eluted from the magnetic particles by methods that break the noncovalent bonds between the component and the biological recognition molecule on the magnetic particles. These methods include, but are not limited to, changes in pH, addition of salt, or addition of chaotropic agents such as guanidinium chloride or sodium dodecyl sulfate. These methods are well known in the art and need not be described further here.

The component to be isolated can be a protein, an antigen, a hapten, a virus, a receptor, a carbohydrate, a hormone, a membrane, an organelle, a bacterial cell, an animal cell, or any molecule or molecular assembly that is specifically bound by the biological recognition molecule. One particularly significant component to be isolated is immune system stem cells. These possess an antigen known as CD34 on their surface and can be selected and isolated by means of antibodies specific for that antigen. Immune system stem cells can be selected and isolated as a means of repopulating the immune system of a patient after extensive chemotherapy or radiation. The cells are isolated before the treatment and reinfused into the patient after the treatment. Preferably, a sufficient quantity of immune system stem cells is purified to repopulate the immune system of a patient who has previously undergone therapy killing the stem cells of the patient.

Conclusion

The magnetic particles of the present invention can be used for simultaneously performing multiple separations and purifications of different components, such as cells bearing different Cluster of Differentiation (CD) antigens. The simultaneous separations are performed in parallel. Other applications which exploit the unusual and controllable magnetic characteristics are also possible.

The foregoing description of the exemplary embodiment of the invention has been presented for the purposes of illustration and description. It is not intended to be exhaustive or to limit the invention to the precise form disclosed. Many modifications and variations are possible in light of the above teaching. It is intended that the scope of the invention be limited not with this detailed description, but rather by the claims appended hereto.

What is claimed is:

1. A liquid solution containing a plurality of discrete magnetic particles, each of the particles having a generally laminar shape and comprising:

a first ferromagnetic layer having a moment oriented in a first direction;

a second ferromagnetic layer having a moment oriented in a second direction generally antiparallel to said first direction; and;

a nonmagnetic spacer layer located between and separating the first and second ferromagnetic layers, the thickness of the laminar-shaged particle being substantially the same as the total thickness of said layers making up the particle; and

wherein the magnitude of the moment of the first ferromagnetic layer is substantially equal to the magnitude of the moment of the second ferromagnetic layer so that the particle has substantially zero net magnetic moment in the absence of an applied magnetic field.

2. The solution of discrete particles of claim 1 wherein each particle further comprises a first antiferromagnetic layer in contact with and exchange coupled with the first ferromagnetic layer for pinning the moment of the first ferromagnetic layer in said first direction.

3. The solution of discrete particles of claim 2 wherein each particle further comprises a second antiferromagnetic layer in contact with and exchange coupled with the second ferromagnetic layer for pinning the moment of the second ferromagnetic layer in said second direction.

4. The solution of discrete particles of claim **1** wherein each particle further comprises a layer of nonmagnetic material for providing the particle with a distinct color.

5. The solution of discrete particles of claim **4** wherein the nonmagnetic material for providing color in each of the particles is Cu.

6. A liquid solution containing a plurality of discrete magnetic particles, each of the particles having a generally laminar shape and comprising:

a first ferromagnetic layer having a moment oriented in a first direction;

a second ferromagnetic layer having a moment oriented in a direction substantially antiparallel to the moment of the first ferromagnetic layer; and

a nonmagnetic spacer layer located between and separating the first and second ferromagnetic layers, the first and second ferromagnetic layers having moments of substantially the same magnitude, whereby the particle has substantially zero net magnetic moment in the absence of an applied magnetic field.

* * * * *

US006432630B1

(12) **United States Patent**
Blankenstein

(10) **Patent No.: US 6,432,630 B1**
(45) **Date of Patent: Aug. 13, 2002**

separator

(54) **MICRO-FLOW SYSTEM FOR PARTICLE SEPARATION AND ANALYSIS**

(75) Inventor: **Gert Blankenstein**, Dortmund (DE)

(73) Assignee: **Scandinanian Micro Biodevices A/S**, Lungy (DK)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **09/254,310** 102(e)

(22) PCT Filed: **Sep. 4, 1997**

(86) PCT No.: **PCT/DK97/00368**

§ 371 (c)(1),
(2), (4) Date: **Dec. 16, 1999**

(87) PCT Pub. No.: **WO98/10267**

PCT Pub. Date: **Mar. 12, 1998**

(30) **Foreign Application Priority Data**

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Feb. 10, 1997 (DK) 0150/97

(51) **Int. Cl.**[7] **G01N 33/553**

(52) **U.S. Cl.** **435/4**; 422/50; 422/51; 422/68.1; 422/82.05; 422/82.08; 422/186; 422/186.1; 435/287.1; 435/287.2; 435/287.3; 436/501; 436/518; 436/526; 436/514

(58) **Field of Search** 422/50, 51, 68.1, 422/82.05, 82.08, 186, 186.01; 435/4, 287.1, 287.2, 287.3; 436/501, 514, 518, 526

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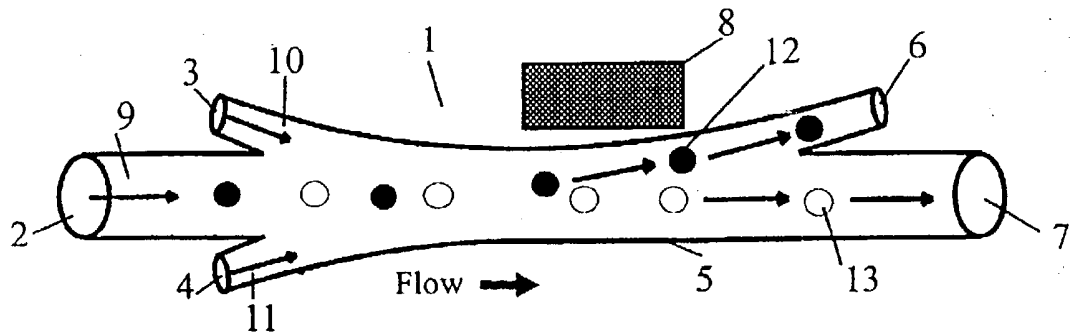
Primary Examiner—Christopher L. Chin
(74) *Attorney, Agent, or Firm*—Merchant & Gould PC

(57) **ABSTRACT**

A micro flow system is provided for separating particles, comprising a microfabricated member having a flow channel (5) defined therein for guiding a flow of a fluid containing the particles through the flow channel, first inlet means (2) positioned at one end of the flow channel for entering the fluid into the flow channel, first outlet means (7) positioned at the other end of the flow channel for discharging the fluid from the flow channel, the flow of the fluid containing the particles being controlled in such a way that one particle at the time passes a cross section of the flow channel, the member being positioned in a field that is substantially perpendicular to a longitudinal axis of the flow channel so that particles residing in the flow channel and being susceptible to the filed across the flow channel are deflected in the direction of the field. Further, a micro flow system is provided for analyzing components of a fluid comprising a microfabricated member having a flow channel defined herein for guiding a flow of a fluid from the flow channel, first inlet means for entering particles into the flow channel, first outlet means for discharging of fluid from the flow channel and a plurality of assay sites located in the flow channel and comprising immobilized reagents whereby the fluid may be analyzed for a plurality of components while residing in the flow channel.

30 Claims, 14 Drawing Sheets



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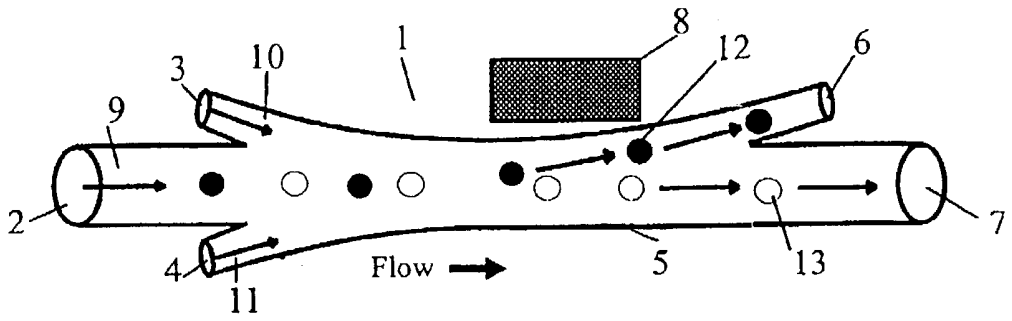


Fig. 1

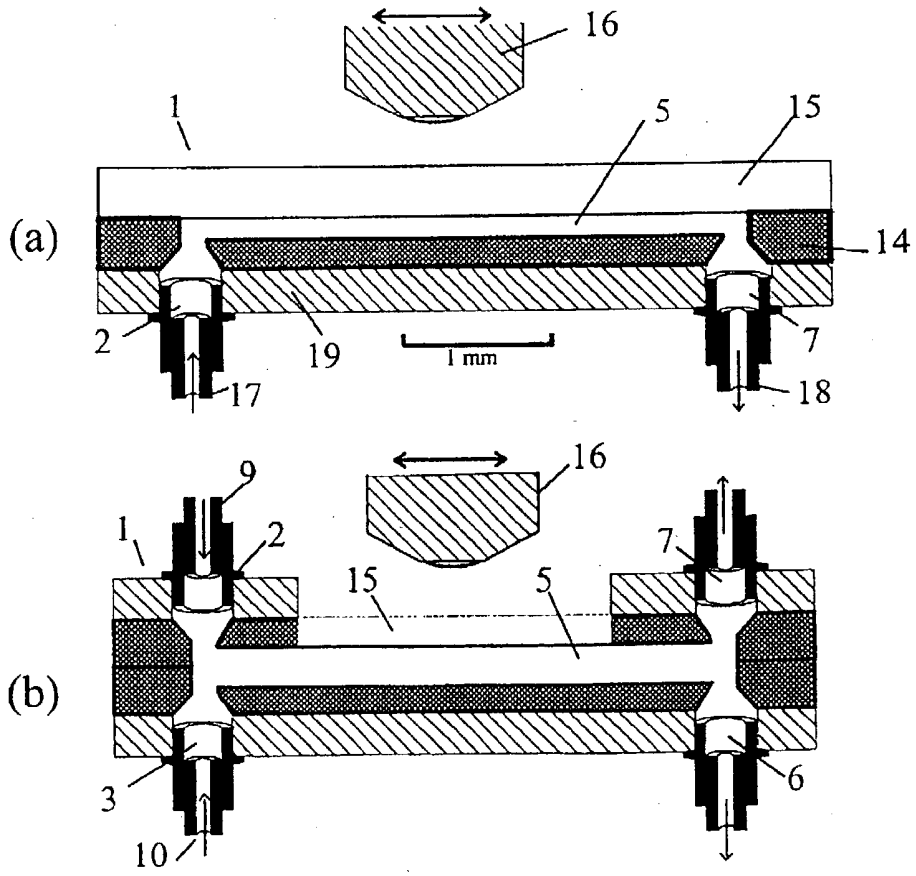


Fig. 2

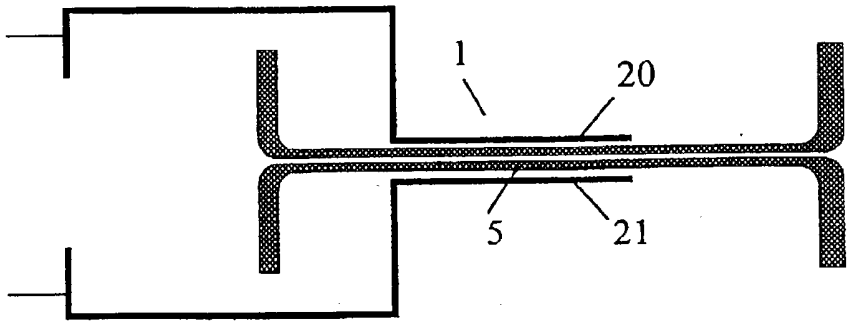


Fig. 3

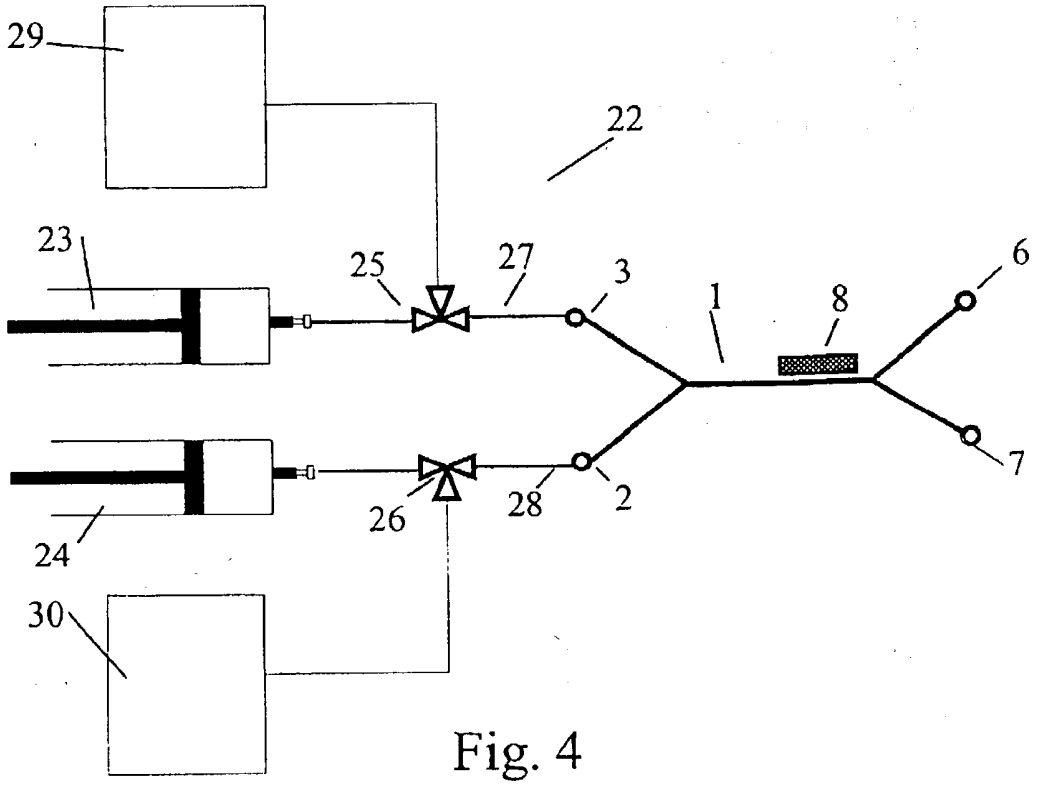
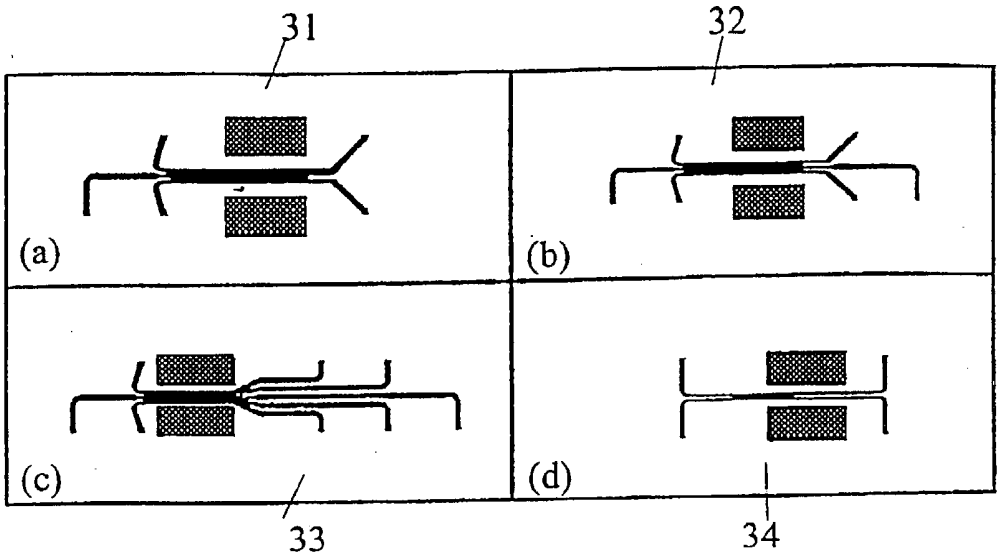


Fig. 4



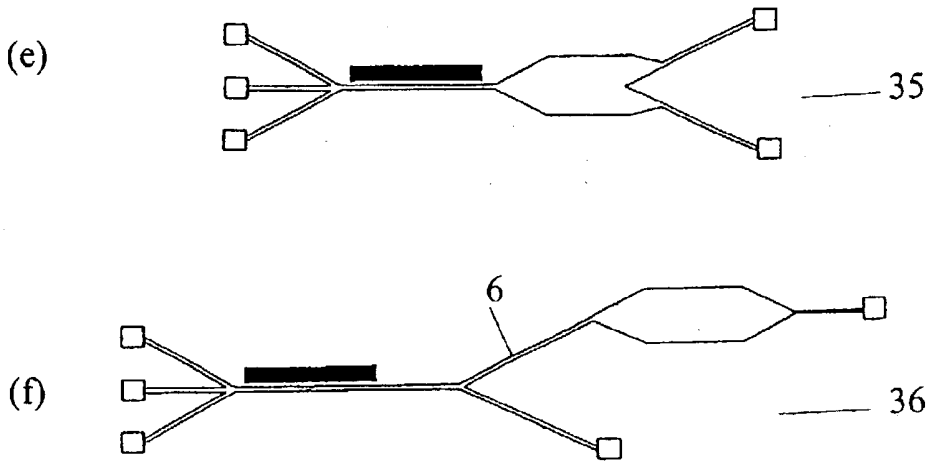


Fig. 5

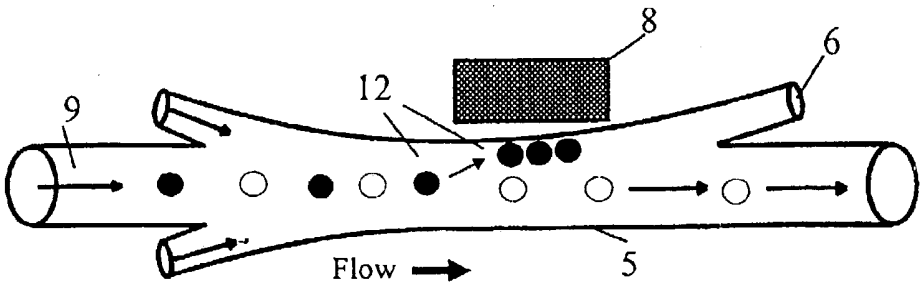

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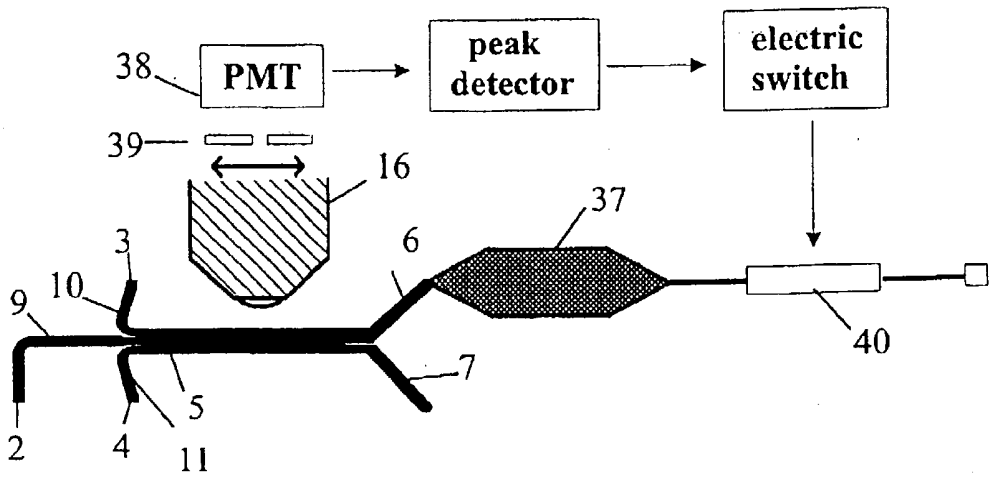
Fig. 6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PMT
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electric switch
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Fig. 7

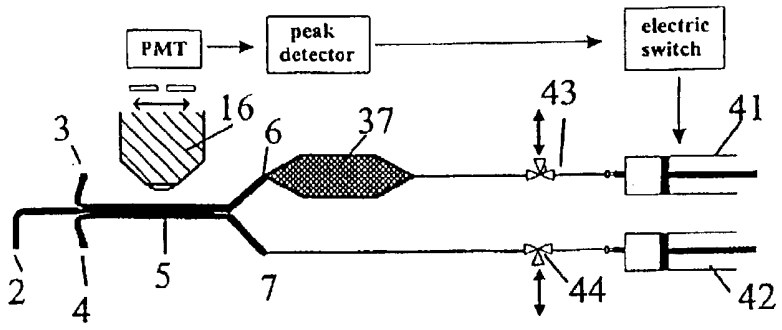
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Fig. 8

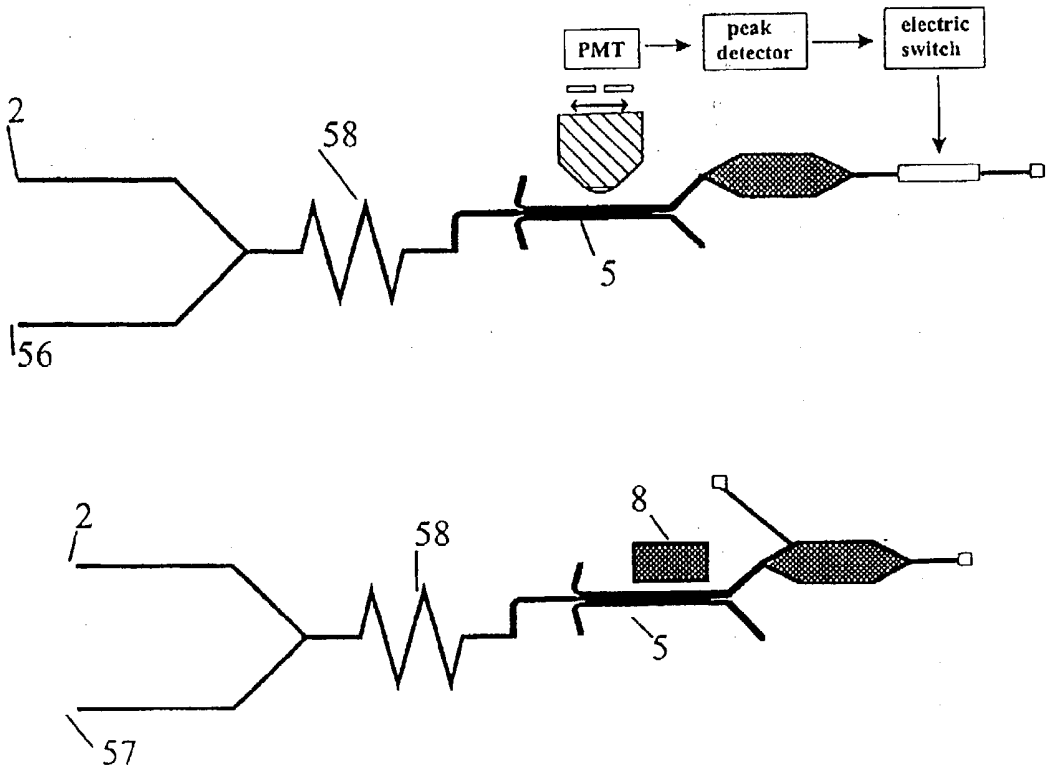
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Fig. 10

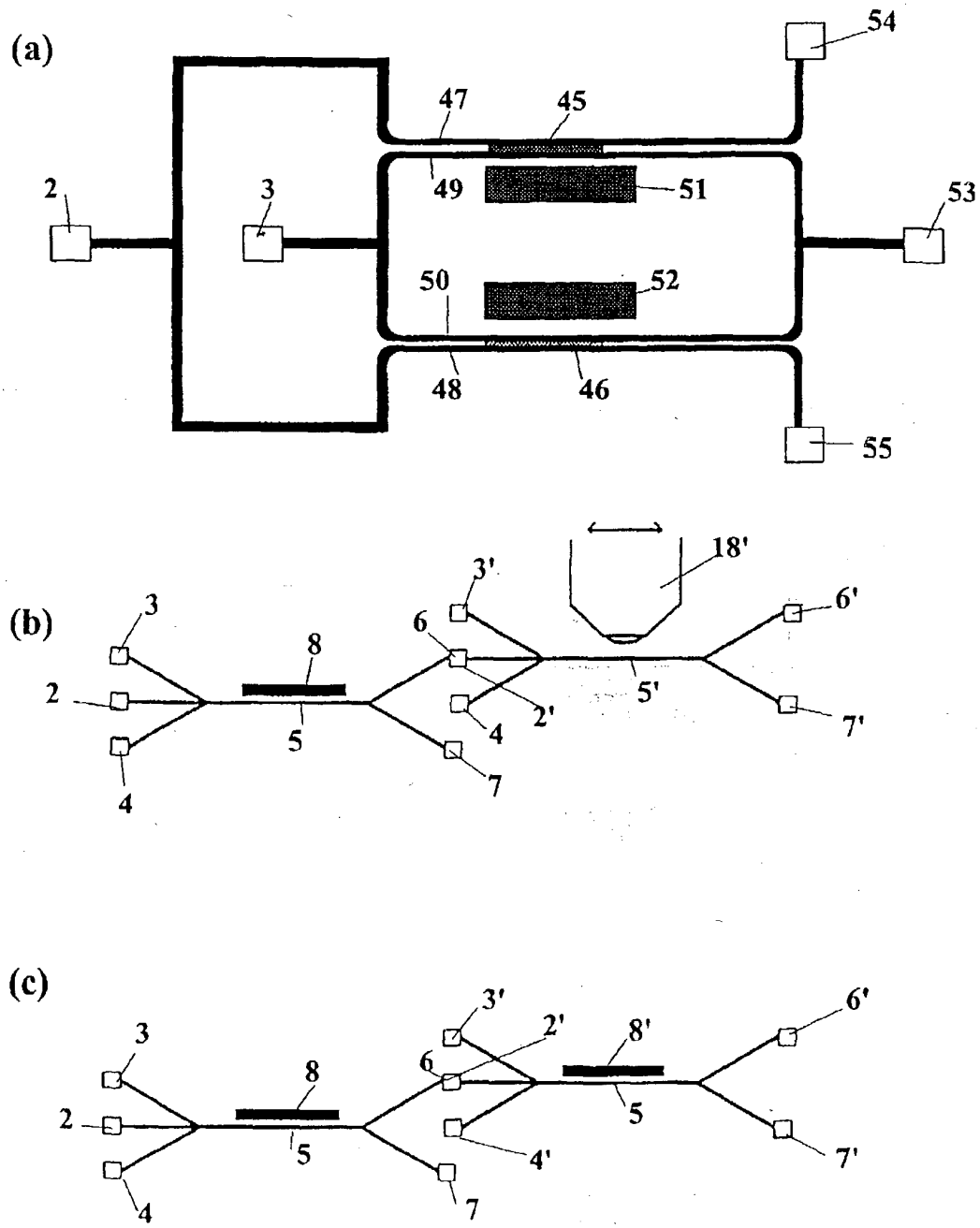
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Fig. 9

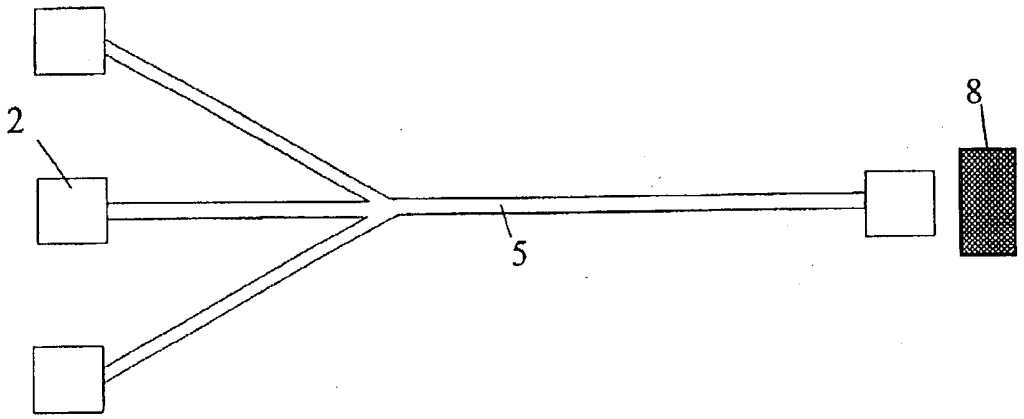

Fig. 11

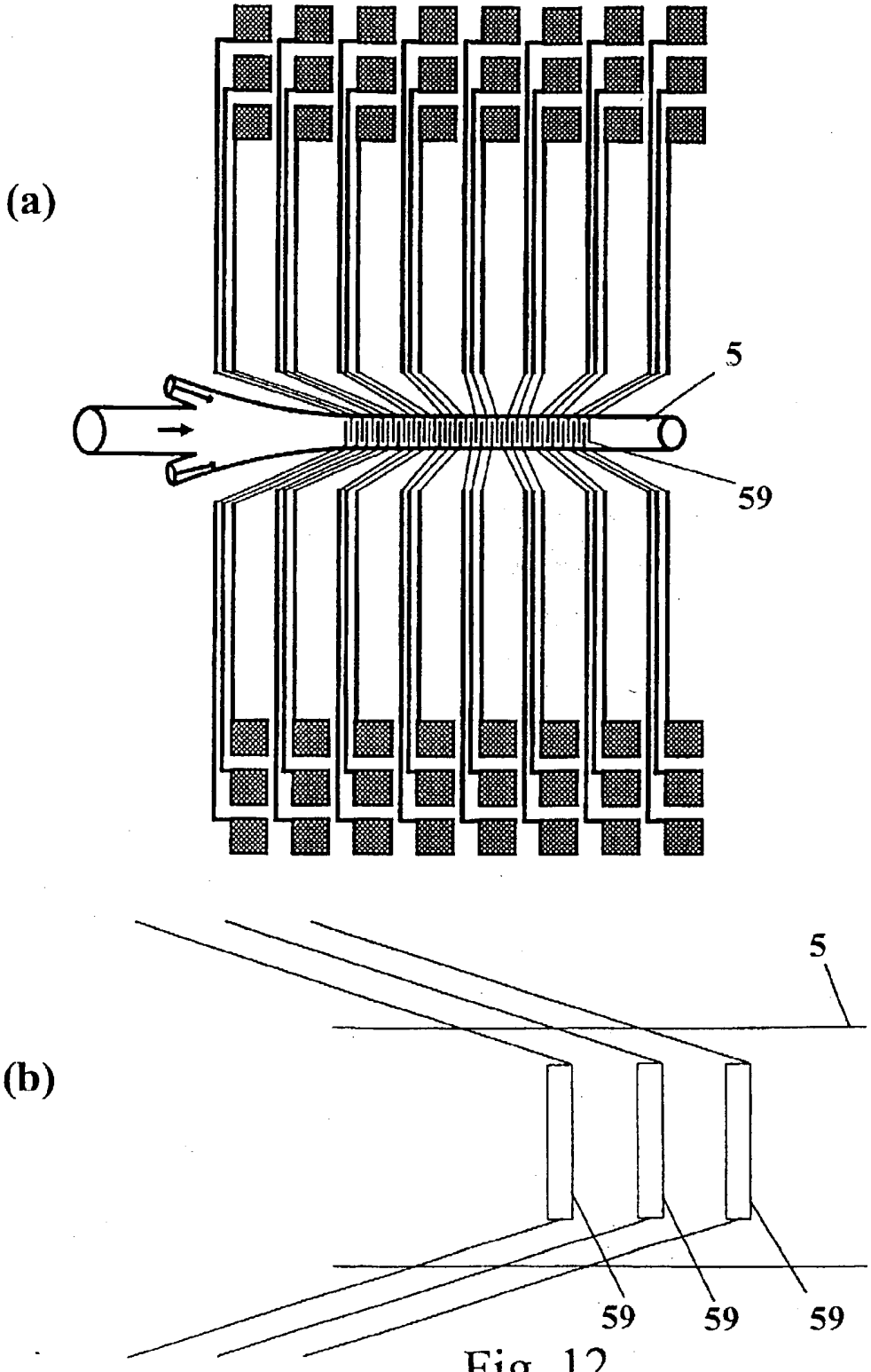

Fig. 12

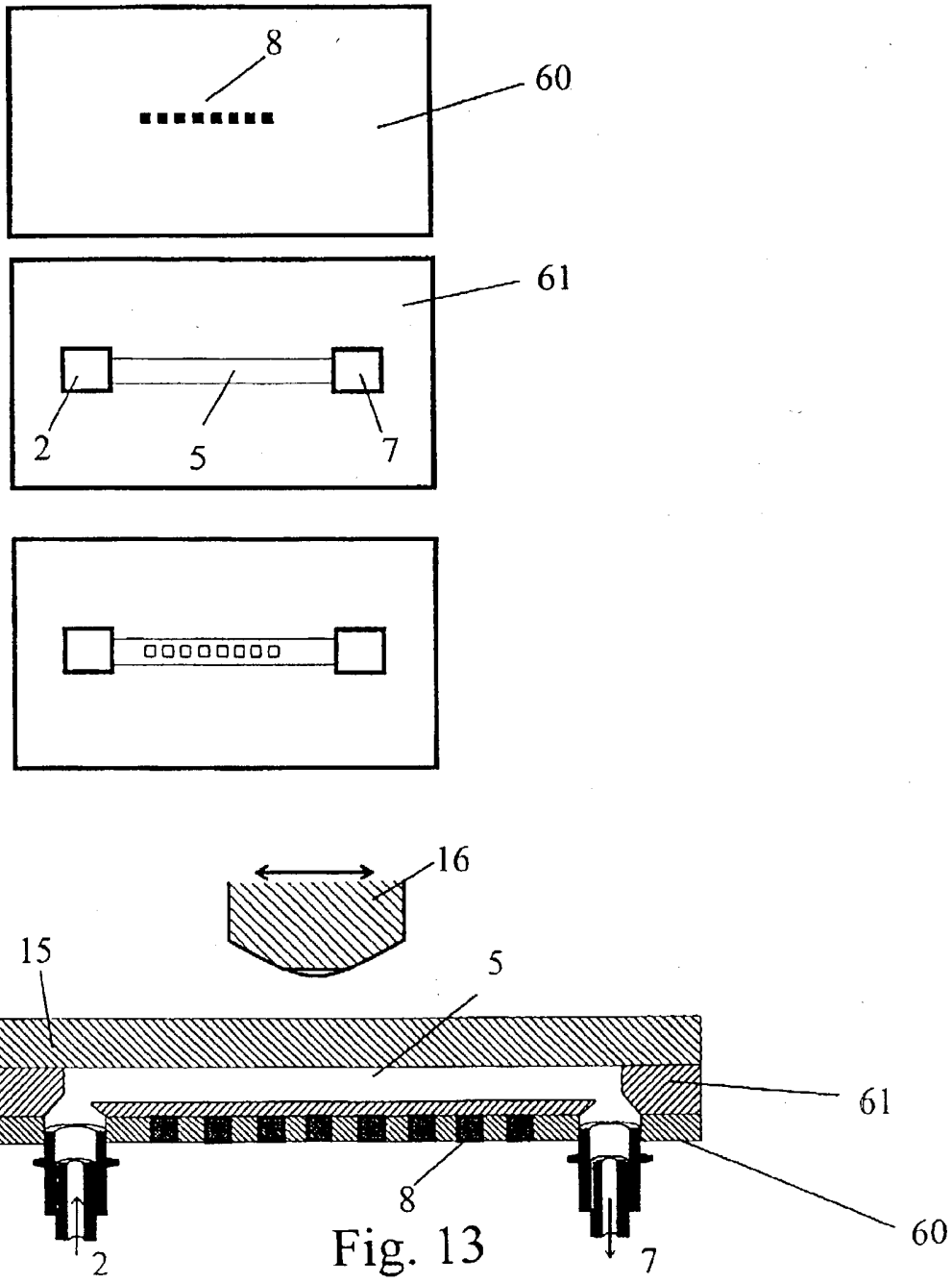

Fig. 13

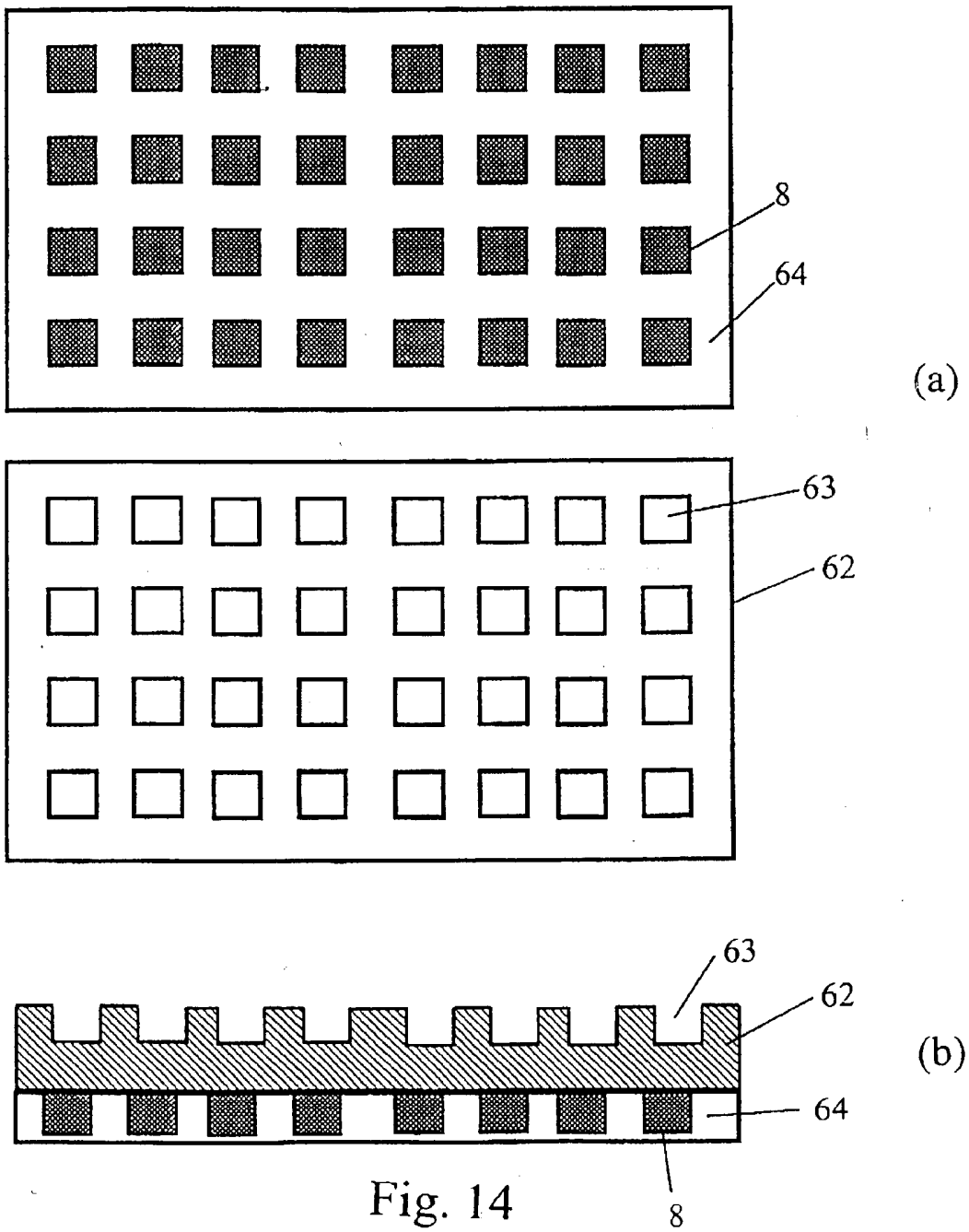

Fig. 14

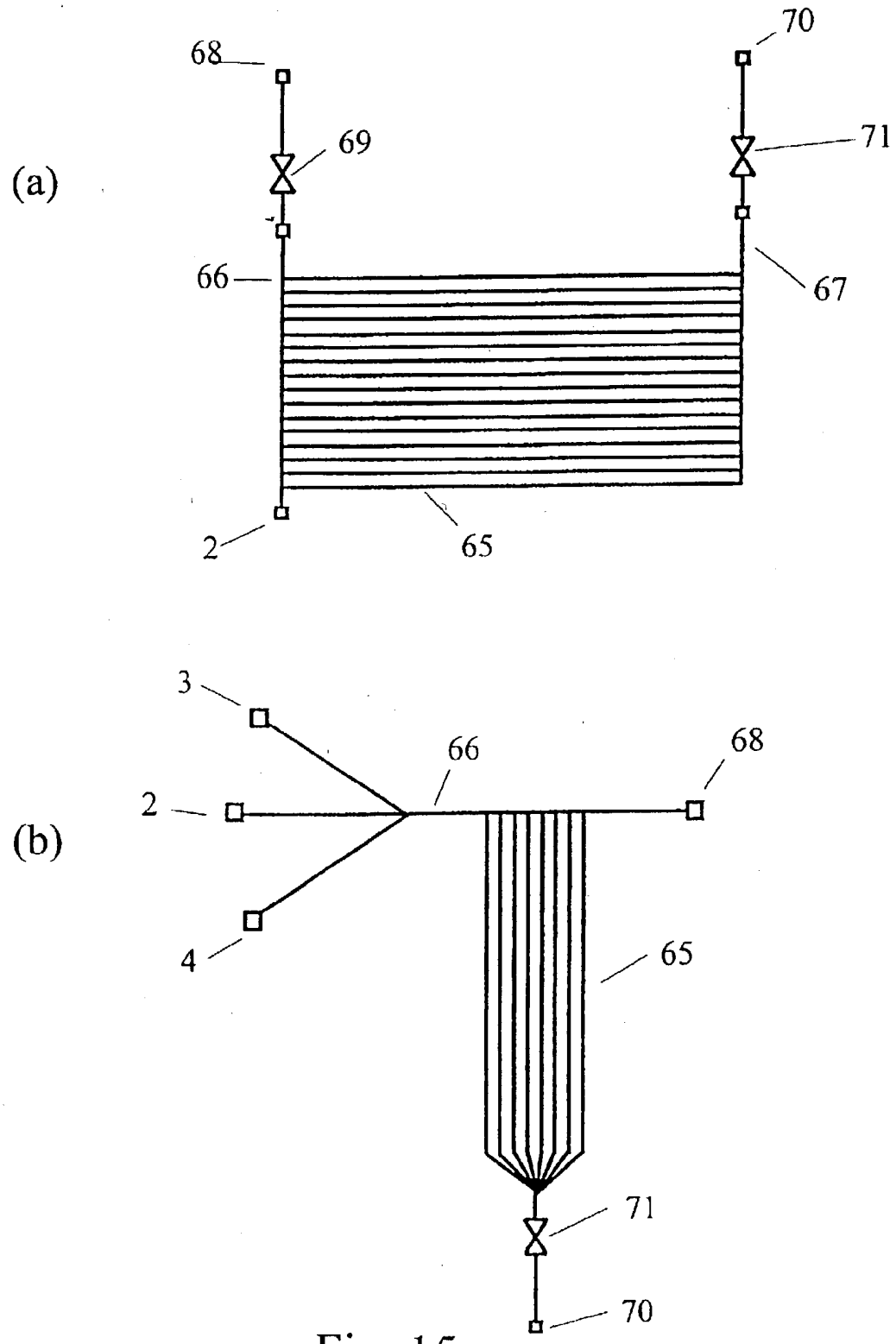

Fig. 15

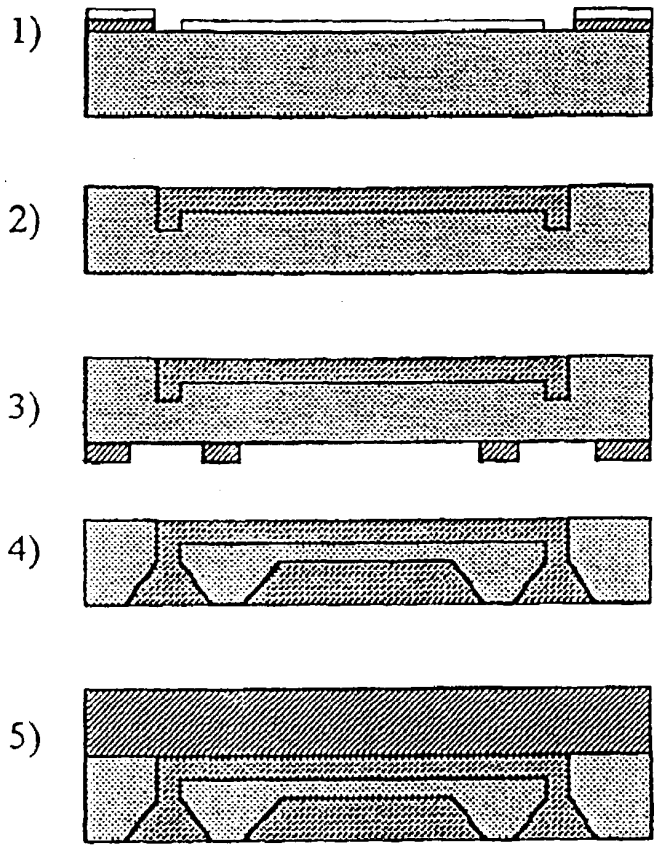

Fig. 16

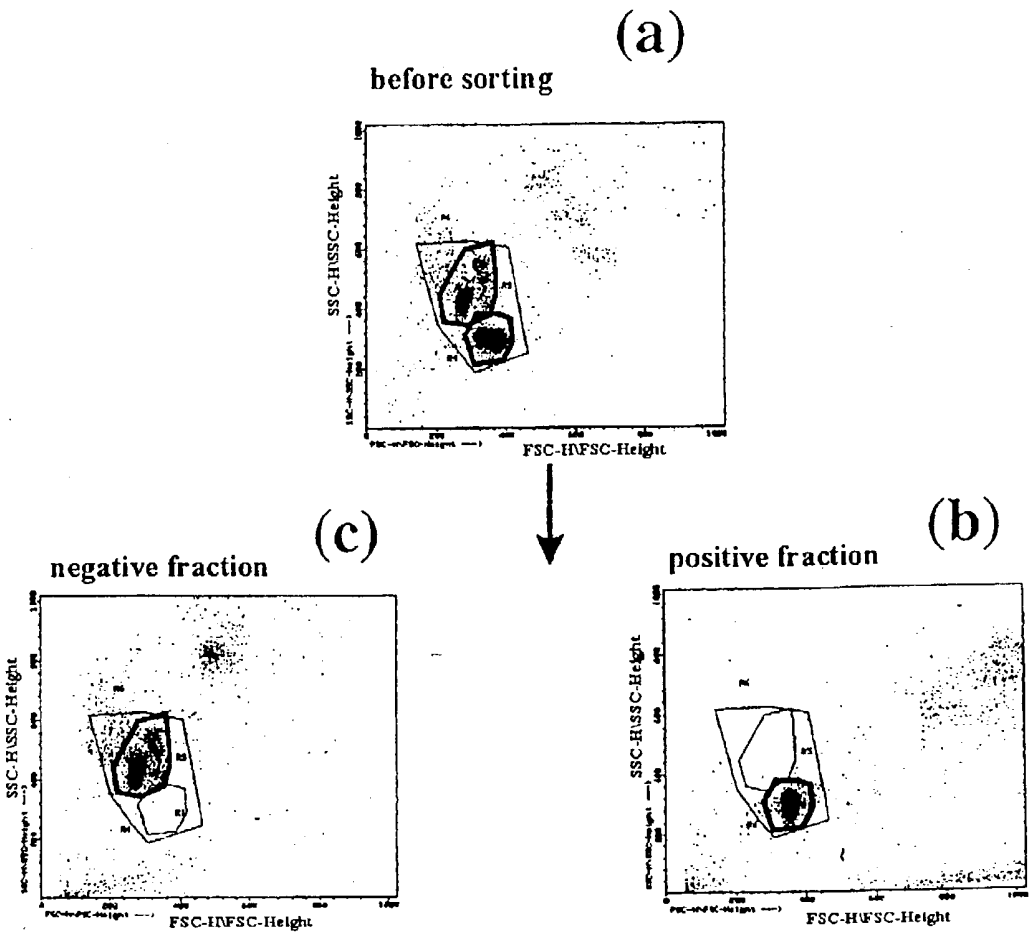
(a)
before sorting
SSC-H\SSC-Height
FSC-H\FSC-Height
(c)
negative fraction
SSC-H\SSC-Height
FSC-H\FSC-Height
(b)
positive fraction
SSC-H\SSC-Height
FSC-H\FSC-Height

Fig. 17

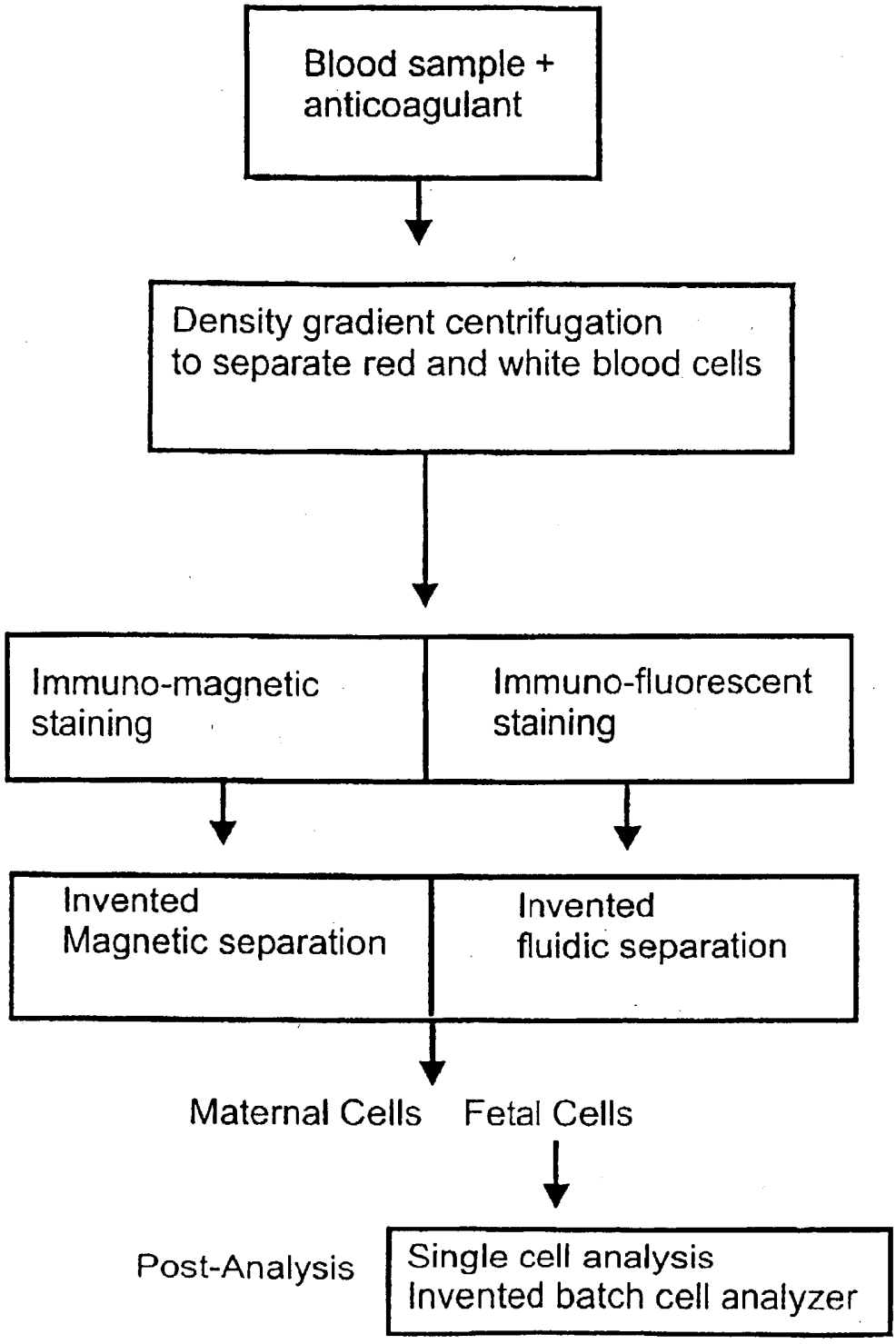
Blood sample + anticoagulant
Density gradient centrifugation to separate red and white blood cells
Immuno-magnetic staining
Immuno-fluorescent staining
Invented Magnetic separation
Invented fluidic separation
Maternal Cells
Fetal Cells
Post-Analysis
Single cell analysis Invented batch cell analyzer

Fig. 18

MICRO-FLOW SYSTEM FOR PARTICLE SEPARATION AND ANALYSIS

FIELD OF THE INVENTION

The present invention relates to methods and apparatuses for detection, separation, sorting, and analysis of particles, such as cells, cell organelles, beads, molecules, such as Deoxyribonucleic acid (DNA), proteins, etc. in a fluid. In particular, the invention relates to particle separation by using different forces such as magnetic, electrophoretic, hydrodynamic and/or gravitational forces, e.g. for utilisation in flow cytometry, light microscopy, electrophoretic separation, magnetophoresis, etc.

BACKGROUND OF THE INVENTION

Flow cytometry is a well known technique that is used for high throughput measurements of optical and/or electrical characteristics of microscopic biological samples. Flow cytometry instruments analyse and isolate cells and organelles with particular physical, biochemical, and immunological properties.

Traditionally, cell sorting by flow cytometry (fluorescence activated cell sorting) has been the method of choice for isolation of specific cell populations by surface markers. However, cell sorting by flow cytometry suffers from several drawbacks, especially high dilution of desired cell sample, low speed and sterility problems. Furthermore, the equipment is very costly with high operation and maintenance cost, making the technique available only to a limited number of laboratories.

During the last few years, isolation of cells by antibody-coupled magnetic beads and carriers has been developed into a reliable tool for the isolation and characterisation of cell populations. Immunomagnetic cell separation, e.g. as commercially introduced by Dynal A/S and Miltenyi Biotec, has become an established method for cell analysis in clinical diagnostics. Due to the relatively low prize, this method is attractive in flow cytometry, especially in sorting of rare cellular events. For example, sorting of fetal cells contained in maternal blood sample provides a non-invasive alternative to prenatal diagnostic procedures, such as amniocentesis of cholionic villus sampling. Another rare event scenario is the detection of low concentration of cancer cells which has an important role in diagnosis of minimal residual disease and evaluation of appropriate therapies. Another medical application for cell sorting systems is the diagnosis of bacterial and viral diseases.

Although this method offers relatively inexpensive approach to sort rare cellular event, it adds considerable time onto the overall rare event detection and it does not offer the multiparameter analysis readily available with flow cytometry techniques. Existing techniques for magnetic separation are suffering from the low purity of the sorted cell fraction and the low recovery rate of the sorted cells. In most systems several washing steps have to be implemented into the separation procedure which then causes cell losses. Additionally small subpopulation of labelled cells cannot be directly isolated by existing magnetic separation techniques.

A good overview about fluorescence activated cell sorting procedures and magnetic activated cell sorting is given in Melamed et. al., "Flow Cytometry and Sorting, (Ed. Melamed et al., Wiley & Sons Inc., 1990).

SUMMARY OF THE INVENTION

Advances in microfabrication and microfluidic technologies continue to fuel further investigation into the miniaturisation of bioanalytical instruments and biochemical assays in general. The present invention relates to development of a low cost non-invasive diagnostic test method and devices for carrying out such tests that include measuring, monitoring, sorting and analysing samples containing particles, such as organic cells, microbeads, cell organells and macromolecules such as DNA. The present invention provides a cheap, fast and reliable method and devices for handling, sorting and analysis of such particles.

Separation may be performed according to various physical properties, such as fluorescent properties or other optical properties, magnetic properties, density, electrical properties, etc. According to an important aspect of the invention, particle separation is performed by aligning the particles in one row of particles in a micro flow channel so that particles can be treated individually.

Thus, it is an object of the present invention to provide a micro flow system and a method of particle separation having an improved efficiency of particle separation compared to the prior art.

It is another object of the present invention to provide a micro flow system and a method for particle separation in which cell lysis is minimised

It is yet another object of the present invention to provide an improved method for preparation of fluids containing particles for separation and analysis of the particles.

It is a still further object of the present invention to provide a micro flow system and a method for simultaneous separation of particles into a plurality of groups of particles.

It is a still further object of the present invention to provide a micro flow system including facilities for pre-treatment and/or post-treatment of a sample.

It is a still further objective of the invention is develop a system for separation and analysis of fetal cells in whole maternal blood samples using an integrated automated micro flow system. The system is designed by downscaling and combining different methods for handling, manipulation and analysis of biochemical samples. Thus, prenatal diagnostics by analysis of fetal cells separated from a whole maternal blood sample is an area, which can benefit from advances in miniaturisation.

It is another objective of the invention is develop a system for separation and analysis of cancer cells from a sample containing cancer cells and healthy cells using an integrated automated micro flow system. The system is also designed by downscaling and combining different methods for handling, manipulation and analysis of biochemical samples. Thus, cancer diagnostics by analysis of cancer cells separated from healthy cells is also an area which can benefit from advances in miniaturisation.

According to a first aspect of the invention the above and other objects are fulfilled by a micro flow system for separating particles, comprising a member having

- a flow channel defined therein for guiding a flow of a fluid containing the particles through the flow channel,
- first inlet means positioned at one end of the flow channel for entering the fluid into the flow channel,
- first outlet means positioned at the other end of the flow channel for discharging the fluid from the flow channel,
- the flow of the fluid containing the particles being controlled in such a way that one particle at the time passes a cross-section of the flow channel,
- the member being positioned in a field that is substantially perpendicular to a longitudinal axis of the flow channel so that particles residing in the flow channel and being

susceptible to the field across the flow channel are deflected in the direction of the field.

According to a second aspect of the invention the above and other objects are fulfilled by a method of separating particles, comprising the steps of

guiding a flow of a fluid containing the particles through a flow channel in such a way that one particle at the time passes a cross-section of the flow channel,

positioning the flow channel in a field that is substantially perpendicular to a longitudinal axis of the flow channel so that particles residing in the flow channel and being susceptible to the field across the flow channel are deflected in the direction of the field and thereby separated from the fluid.

According to a third aspect of the invention the above and other objects are fulfilled by a micro flow system for separating particles, comprising a member having

a flow channel defined therein for guiding a flow of a fluid containing the particles through the flow channel,

first inlet means positioned at one end of the flow channel for entering the fluid into the flow channel,

first and second outlet means positioned at the other end of the flow channel for discharging of fluid from the flow channel,

the flow of the fluid containing the particles being controlled in such a way that one particle at the time passes a cross-section of the flow channel,

monitoring means positioned at the flow channel for monitoring parameters of a particle residing within a measurement volume within the flow channel and providing an output signal corresponding to a monitored parameter,

control means for controlling passage of fluid through the first and the second outlet means, respectively, in response to the output signal of the monitoring means whereby particles may be separated according to values of a parameter monitored by the monitoring means.

According to a fourth aspect of the invention the above and other objects are fulfilled by a method of separating particles, comprising the steps of

guiding a flow of a fluid containing the particles through a flow channel in such a way that one particle at the time passes a cross-section of the flow channel, the flow channel having first and econd outlet means for discharging of fluid from the flow channel, monitoring parameters of a particle residing within a measurement volume within the flow channel and

controlling passage of fluid through the first and the second outlet means, respectively, in response to a monitored parameter value whereby particles may be separated according to values of a monitored parameter.

According to a preferred embodiment of the invention, a method of separating fetal cells from maternal cells, comprising the steps of selective magnetically staining of fetal cells in a fluid containing fetal and maternal cells, guiding a flow of the fluid containing the fetal cells through a flow channel in such a way that one fetal cell at the time passes a cross-section of the flow channel, positioning the flow channel in a magnetic field that is substantially perpendicular to a longitudinal axis of the flow channel so that magnetically stained fetal cells residing in the flow channel are deflected in the direction of the magnetic field.

Further a method is provided for separating cancer cells from other cells, comprising the steps of selective magnetically staining of cancer cells in a fluid containing cancer and other cells, guiding a flow of the fluid containing the cancer cells through a flow channel in such a way that one cancer cell at the time passes a cross-section of the flow channel, positioning the flow channel in a magnetic field that is substantially perpendicular to a longitudinal axis of the flow channel so that magnetically stained cancer cells residing in the flow channel are deflected in the direction of the magnetic field.

The particles to be separated from other particles in a fluid and/or to be separated from the fluid containing the particles may comprise living cells, chromosomes, organelles, beads, biomolecules, such as Deoxyribonucleic acid (DNA), proteins, etc.

Preferably, the flow through the flow channel is a laminar flow so that flow of particles are predictable and easy to control, e.g. with a flow of guiding buffers.

When the flow is laminar, the stream of particles can be positioned as desired within the flow channel, e.g. by controlling flow velocities of the fluid containing particles at the particle inlet of the member and flow velocities of guiding buffers at corresponding inlets.

Preferably, the flow channel is small for the flow through the channel to have a low Reynolds number, e.g. in the range of 0.01–500, such as 0.05–50, preferably 0.1–25. Thereby, inertial effects, which causes turbulence and secondary flows are negligible, viscous effects dominate the dynamics, and mixing is caused only by diffusion. Flow of the sample, which is the fluid containing particles and guiding buffers can be laminated in guided layers through the channel and displacement of particles in the channel is only caused by the external force applied. The Reynolds number referred to is based on the hydraulic diameter of the flow channel, the flow velocity in the axial direction and the fluid density and viscosity, $Re=\rho Dh/\mu$ where the hydraulic diameter Dh is defined as four times the cross-sectional area divided by the wetted perimeter.

The illustrated flow channels of the micro flow system have a width ranging from 0.1 to 0.55 mm, preferably ranging from 0.1 to 0.4 mm, in particular ranging from 0.1 to 0.2 mm, and a depth ranging from 0.04 to 0.2 mm, preferably ranging from 0.04 to 0.1. With respect to the lowest cross-sectional area of the flow channel, it is preferred that this area is in the range of 0.004 to 0.11 mm^2, in particular in the range of 0.004 to 0.02 mm^2.

It is believed that any length of the flow channel within the range of 0.1 to 20 mm, preferably 1.0 to 3.5 mm, would lead to satisfactory results.

Preferably, the system is operating with total volumetric flow rates of 0.1 up to 200 μl/min, which gives a flow velocity of 15 mm/min up to 180 mm/min. The average residence time of a particle inside the flow channel, which corresponds to a separation time ranging from 0.1 to 6 sec. The residence time of the sample is defined by the total volumetric flow rate of the system.

The micro flow system may comprise flow rate adjustment means for adjustment of the time the particles reside in the flow channel.

Preferably, the fluid channel is sized so that for efficient separation, particles are displaced 10–30 μm in the flow channel. Thereby, the particle may only be exposed to a field for a very short period of time and thus, continuous separation of particles may be facilitated.

In order to collect the particles, which are deflected in the flow channel, the micro flow system may further comprise second outlet means for discharging particles having been deflected in the flow channel.

The micro flow system may comprise second inlet means for entering a first guiding buffer into the flow channel together with the fluid containing particles. When the flow is laminar, the two fluids flow through the flow channel in parallel abutting each other along a small area extending along a longitudinal axis of the flow channel whereby the cross-section and the path through the flow channel of the flow of the fluid containing particles may be controlled by the first guiding buffer flow. Further, particles in the fluid containing particles may be deflected into the guiding buffer fluid when the two fluids pass the field essentially perpendicular to the longitudinal axis of the flow channel. Furthermore, two (or even more) outlets may be provided at the down stream end of the flow channel for discharging the guiding buffer now containing separated particles and fluid substantially without particles susceptible to the field essentially perpendicular the flow channel, correspondingly.

The micro flow system may further comprise third inlet means for entering a second guiding buffer for improved control of the path of particle flow through the flow channel. By adjustment of the flow velocities of the guiding buffers and the fluid containing particles, the flow within the flow channel of fluid containing particles may be controlled to flow within an essentially cylindrical shaped domain with a longitudinal axis extending substantially parallel to a longitudinal axis of the flow channel and further the position within the flow channel and the diameter of the flow cylinder may be controlled by corresponding adjustments of the volumetric ratio between the flow rate of the fluid containing particles and the flow rate of the guiding buffers.

It is possible to control the cross-sectional area of the domain containing the sample to be a little larger than the cross-sectional area of the particles by adjusting the volumetric flow rates of the sample and of the one or two guiding buffers in such a way that the particles contained in the sample are aligned in a single row of particles. This is a very important feature since it enables individual treatment of each particle and it leads to a sensitive method of sorting particles according to their susceptibility to a field. A sample flow layer thickness less than 1 μm may be achieved.

Preferably, the channel depth is small enough, e.g. below 50 μm, to allow observation of the particles flowing through the channel by a microscope. In an important embodiment of the present invention, the micro flow system comprises a cover, e.g. a transparent or translucent cover, for covering the flow channel. When the cover is transparent or translucent, it will be possible to observe events in the flow channel, e.g. passage of a stained or coloured particle or cell.

The member with the flow channel may be produced from any suitable material, such as silicon, polymers, such as Plexiglas, Teflon, etc., glass, ceramics, metals, such as copper, alumna, stainless steel, etc., etc.

The channel may be provided in the member by any suitable manufacturing process, such as milling, etching, etc.

In a preferred embodiment of the invention, the member is a silicon chip manufactured utilising photolithography and the channel is etched into the silicon chip.

The field may be a magnetic field, an electric field, a gravity field, etc., and any combination of such fields.

A magnetic field may be generated by permanent magnets, such as rare earth magnets, such as samarium-germanium magnets, a mixture of ferromagnetic powder and epoxy, etc., etc., electromagnets, e.g., in silicon integrated electromagnets, etc. The magnets are preferably positioned adjacent to the flow channel so that the magnetic field is substantially perpendicular to a longitudinal axis of the flow channel.

In a preferred embodiment of the invention, the magnets are positioned in and glued to rectangular slots that are etched into a silicon chip. The slots are located adjacent to the separation flow channel. In the example shown in FIG. 1, a permanent magnet or an electromagnet can be received by slots in the micro flow system. The slots are, e.g., 0.5 mm wide, 0.5 mm long and 0.2 mm deep. For generation of a magnetic field, a solid magnetic block, i.e. rare earth magnet can be glued into the slot. Alternatively, a mixture of ferromagnetic powder and epoxy can be injected into the slots to produce a high magnetic field gradient.

The strength of the magnetic field inside the micro flow system may be adjustable. If an electromagnet is used for generation of the magnetic field, the magnitude of the field may be varied by varying the amplitude of the voltage input to the electromagnet. If a permanent magnet generated the magnetic field, the magnitude of the field may be varied by varying the distance between the magnet and the flow channel of the micro flow system.

As already mentioned, the net displacement of a particle in the micro flow system depends on the force applied to it by the field. This can be utilised for separation of a first group of particles of various types in a fluid into a plurality of set of particles; each set comprising a specific type of particles. A micro flow system with e.g. five separation outlets may be used to separate a fluid containing particles into five sets of particles, each set comprising particles that are influenced by the field with a force of a specific magnitude, in the following denoted particles with a specific F-value. Particles with a low F-value are only deflected by a small amount by the field and are discharged from the flow channel through a corresponding outlet port. Particle deflection is increased with increasing F-values whereby such particles are discharged from the flow channel through the corresponding other outlets.

The particles to be separated from other particles in a fluid, and/or to be separated from the fluid containing the particles, may be magnetically stained to facilitate separation in a magnetic field.

In the present context, the term staining is to be understood in a broad sense. The term is intended to cover any way of marking a particle thereby facilitating detection of the particle. For example a cell may be stained with a fluorescent substance, such as acridin orange, methylene blue, etc, facilitating detection of the stained particles by a fluorescence detector, or, a particle is said to be magnetically stained when it is coupled to a magnetic microbead. The microbead may for example carry a monoclonal or polyclonal antibody on its surface for coupling to an antigene of a cell to be separated utilizing a magnetic field.

In the case where particles have to be detected in a flow channel by optical means, such particles are preferably stained with a chromophoric reagent, or, a fluorescent probe.

An electric field may be generated by electrodes, such as metal electrodes, such as gold electrodes, etc. The electrode may be positioned inside the flow channel, e.g. to introduce electrophoretic forces, e.g. for separation of charged molecules in the fluid, or outside the flow channel e.g. to introduce dielectrophoretic forces, e.g. for separation of particles contained in the flow according to the susceptibility of the particles to the field. Preferably, the electrodes are positioned in such a way that the electric field is essentially perpendicular to a longitudinal axis of the flow channel.

The electric field may be a high frequency field, e.g. a 5 MHz field generated by electrodes positioned inside the flow channel. Living cells positioned in an electric field will be polarized and will be influenced by the field and thus, an

alternating field may be used to separate living cells from other particles.

The field generated across the flow channel may be utilised for immobilisation of particles whereby particles may be held in substantially fixed positions within the flow channel for a specific period, e.g. as outlined in FIG. **6**, allowing chemical reactions with the particles to take place and/or kinetic measurements on the particles to be performed and/or to bring the particles into contact with different chemical substances or for separating the particles from the sample. The particles may undergo a washing step before removal or reduction of the field redisperses them.

According to a fifth aspect of the invention the above and other objects are fulfilled by a micro flow system for separating particles, comprising a member having

a flow channel defined therein for guiding a flow of a fluid containing the particles through the flow channel,

inlet means positioned at one end of the flow channel for entering the fluid into the flow channel,

field generating means positioned proximate to the other end of the flow channel for generating a field substantially along a longitudinal axis of the flow channel whereby the particles are drawn by the field along the channel and distributed according to their susceptibility to the field and their mobility.

For example, means for generating a magnetic field may be situated at the closed end of a micro flow channel, which at the other end has at least one inlet for entering a sample containing magnetic labelled macromolecules, i.e. ribonucleic acid or proteins. The sample is entered into the channel where the particles are drawn by the magnetic field along the channel and, as by electrophoresis, the particles will be distributed according to their susceptibility to the magnetic field and their mobility. The generated magnetic field is removed after a predetermined time interval and the distribution of particles can then be observed.

According to another embodiment of the invention, the flow through the sort outlet is not continuous but only allowed by a controlling means, e.g. a valve, when a particle with the desired characteristics is detected by a detection means. The particles are sorted using hydrodynamic forces in the sense that the flow is diverged from the ordinary outlet to the sort outlet only when it contains a particle that fulfils certain criteria. The concentration of sorted particles in the flow out of the sort outlet will consequently be high. This is especially an advantage for sample flow with rare occurrence of particles that are searched for. The detection means can be e.g. optical detection means or magnetic detection means e.g. a Hall sensor or means for detecting e.g. electrical or other properties of the particles. The detection means can in an alternative embodiment be used for counting of particles with the desired characteristics as a separate function or in connection with any of the other embodiments described herein.

In yet another embodiment, the field strength is adjustable, e.g. by adjusting the voltage supplied to an electromagnet or to a set of electrodes or by adjusting the distance from a permanent magnet to the flow channel. Particles are in a first operation mode entrapped inside the flow channel by the field at high intensity while at the same time the sort outlet is closed. In a second operation mode, the field is reduced and the sort outlet is open in such a way that the entrapped particles are redispersed and moved out of the sort outlet. Particles that are rare in the sample can by switching between these two operational modes be sorted out in a highly concentrated form An example of this embodiment is outlined in FIG. **6**.

In a further interesting embodiment, the micro flow system according to the invention involves facilities for performing pre-treatment and/or post-treatment of the fluid comprising the particles. These possibilities are outlined in FIGS. **5***(f)*, **7** and **10**. As an example, the particles may be treated with a reagent before entering the flow channel, e.g. undergo magnetic or chromophoric staining. Post-treatment may comprise means for collecting or concentrating the deflected particles or means for contacting the deflected particles with one or more reagent(s).

By one possible combination of the pre-treatment and the post-treatment facilities, cells may undergo magnetic staining before entering the flow channel, and after separation the staining may be removed by treatment of the stained cells with a suitable reagent.

It is an important advantage of the present invention that a micro flow system is provided that operates continuously with no requirement for operator intervention.

It is another advantage of the present invention that separation may be performed in one step.

It is still another advantage of the present invention that the particles can be separated in a continuous flow without substantially interfering with the flow itself and that separated particles may be collected at corresponding separated outlets of the flow channel without having to interrupt the flow in the flow channel.

It is another important advantage of the invention that the particles contained in the sample by the adjustment of the flow rate of one or more guiding buffers can be lined up in one row such that the particles can be analysed and sorted individually. This results in a sorting system with the highest sensitivity to the susceptibility of the single particle to the field applied to the sorting channel and a sorting system with the highest resolution of the detection means of the characteristics exhibited by the particles.

It is yet another advantage of the present invention that the micro flow system is easily integrated into other continuous flow systems, such as flow cytometers, flow injection analysis systems, etc.

It is a further advantage of the present invention that particles may be separated into a plurality of groups of particles, e.g. different subpopulations of cells, based on different susceptibility to the field generated across the flow channel of the different groups of particles. This may be obtained by using a multiple outlet micro flow system as outlined in FIG. **5***(c)*.

It is a still further advantage of the present invention that the micro flow system allows observation of particles in the flow channel using a microscope.

It is a still further advantage of the invention that a closed system is provided allowing biohazardous samples, such as samples containing pathogens, to be entered into the system without contaminating the laboratory environment and without causing hazard for operators working with pathogen biomaterials.

It is a still further advantage of the invention that a system with a low shear stress in the flow is provided allowing a gentle treatment of biological samples; e.g. fragile living cells, especially when two guiding buffers are introduced in the channel.

It is a still further advantage of the invention that a high concentration of the sorted particles can be obtained even from samples with rare occurrence of particles that are searched and sorted for.

According to an important aspect of the invention, a new system for immunomagnetic cell separation and manipulation is provided that utilises a silicon based micro fabricated

flow chip. The system combines the advantage of flow cytometry and immunomagnetic separation technique. The flow chip will be an important component of a portable micro system for cell sorting and analysis. The flow chip is designed for rapid immunomagnetic cell separation nearly without any pressure drop. Its simple and cheap fabrication and versatile sorting and detection properties offer an alternative to conventional cell separation systems.

It is an advantage of the invention that a micro flow system is provided that is cheap, easy to operate, versatile, simple and portable and that offers the possibility of automation.

A miniaturised flow channel system is provided that utilises the advantageous fluid behaviour in micro systems. The invented system operates continuously. Instead of holding back the magnetisable particles in the separation unit, the particles are deflected into the direction of the magnetic field while passing it continuously. By splitting the fluid flow into two or more outlets, the deflection of the particles can be used for separation of particles into different sets of particles, each of which leaves the flow channel through a specific outlet.

The continuous separation system (CSS) allows efficient enrichment as well as depletion of labelled sample contents of interest. The CSS is designed to fit under a microscope allowing parallel detection of the optical properties of the sample and the control of separation of particles.

An advantage of the geometry of the invented separation flow channel is that a magnetised or electrically charged particle has to be moved only over a distance of 10–30 μm to be separated from the fluid containing particles.

Furthermore, the invention enables isolation of multiple cell or particle subpopulations from a single sample at the same time. The magnitude and direction of the force F on a magnetisable particle, e.g. a magnetically labelled cell, is dependent on the magnitude of the magnetic field and the number of magnetic moments inducible on a labelled cell.

$$F=N*S*\mu B*\text{grad } B$$

where S is the number of Bohr magnetons (μB) per particle and N is the number of particles per cell.

Beads with small S are moving a less distance in lateral direction in relation to the flow through the flow channel than beads with a higher S value. This can be used to separate subpopulation of cells labelled with different magnetisable beads: By splitting the flow channel in various outlet channels cells can be distinguish and separated due to their individual F values.

The drag force on a spherical particle can be found from the particle Reynolds number, based on particle diameter, particle velocity relative to the fluid and fluid viscosity and density. In a flow with a Reynolds number less than 100, the drag force D on the particle can be found from a modified version of Stokes law

$$D=3\pi\mu\, Ud(1+3/16\text{Re})^{1/2}$$

where μ denotes the viscosity of the liquid, U is the relative velocity of the particle and d is the diameter. The numerical value of the parenthesis on the right hand side of the above formula is close to unity for Reynolds numbers less than one why it in that case can be omitted. The magnitude of the drag force on the particles, the force applied to the particle by the field, the distance the particle needs to be moved and the time available for the separation are all important aspects to be considered when a separation process and the device for carrying it out is designed.

An example is given for separation by gravitational means. The effective gravitational force G defined as the gravitational force minus the buoyancy force is found as

$$G=(\rho_{particle}-\rho_{liquid})g\pi/6d^3$$

where g is the gravitational constant. For simplicity, a Reynolds number for the particle of less than one is assumed why the drag force D is given in a simple form. These two forces, D and G, are equal when the maximum velocity, the settling velocity U_∞ has been reached. This velocity is found as

$$U_\infty = \frac{(\rho_{particle}-\rho_{liquid})gd^2}{18\mu}$$

The velocity to a given time t can be found as

$$U(t)=U_\infty(1-e^{-1}g/U_\infty)$$

For a particle submerged in water with a diameter of 30 μm and a density of 1.2 times the density of water the settling velocity is 9×10^{-5} m/s. The particle will reach 90% of this velocity after 2.1×10^{-5} seconds why the transient phase can be neglected. It will take the particle 0.33 seconds to travel a distance of 30 μm, which makes the method reasonable to employ for separation purposes.

While instrumentation in chemistry and biochemistry has become more automated in recent years, the preparation of samples remains a highly laboratory intensive task. The demand is increasing for high throughput, easier to use cost effective analytical devices and assays. Creating this opportunity is e.g. the world-wide effort to sequence the Human Genome, resulting in the development of new DNA diagnostics and therapeutics. Another important trend is the minimization of health care costs and hospital admissions by testing patients and monitoring therapeutic use in less expensive environments, the so-called point-of-care analysis.

Micro flow devices containing arrays of nucleic acid hybridisation sites, known as genosensors, are being developed for a variety of uses in genomic analysis. A great deal of the overall genosensor development effort involves optimisation of experimental conditions in the actual use of genosensors.

Another embodiment of the invention is dealing with a low-tech form of genosensor and immunosensor technology, involving arrays of oligonucleotides on a microchip, which can be used to define optimal operating conditions and to develop applications of hybridisation arrays in genome mapping and sequencing. The genosensor array is placed in a micro flow channel system allowing an operation in a flow-through mode. Thus several steps of microliquid handling, e.g. washing and staining steps, reagent addition, can be integrated as an automated routine procedure. Additionally, micro flow devices containing arrays of antibody/antigen sites, known as immunosensors, can be designed in the same way. The system could be used for combinatorial screening (high-throughput screening) and pharmacokinetic studies.

According to a sixth aspect of the invention the above and other objects are fulfilled by a micro flow system for analysing components of a fluid, comprising a member having a flow channel defined therein for guiding a flow of a fluid through the flow channel, first inlet means for entering particles into the flow channel, first outlet means for discharging of fluid from the flow channel and a plurality of assay sites located in the flow channel and comprising

immobilised reagents whereby the fluid may be analyzed for a plurality of components while residing in the flow channel.

The system may further comprise field generating means positioned proximate to at least some of the assay sites for generation of a field proximate to the corresponding assay site whereby reagents residing in the flow channel and being susceptible to the field when generated at a selected assay site are attracted to and immobilised at the selected assay site, or, are rejected from the selected assay site.

In an embodiment of the invention, the member comprises a plurality of flow channels arranged in parallel or in series and each of which has assay sites whereby the fluid containing particles is brought into contact with a large number of assay sites.

According to a seventh aspect of the invention, a method of analysing components of a fluid is provided, comprising the steps of entering a fluid containing the particles into a flow channel and allowing the fluid to flow in the channel, the channel having a plurality of assay sites, each of which comprises immobilised reagents whereby the fluid can be analyzed for a plurality of components while residing in the channel.

According to a eighth aspect of the invention, a method of forming assay sites comprising immobilised reagents in a flow channel is provided, the method comprising the steps of

preparing selected surfaces of the assay sites for immobilisation of selected reagents,

dispensing a selected reagent proximate to a corresponding selected assay site, and

generating a field proximate to the selected site whereby the reagent is attracted towards and brought into contact with the surface of the selected assay site by the field generated and is immobilised upon contact with the surface.

Thus, the micro flow system of the previous section with a flow channel with assay sites may further comprise field generating means positioned proximate to at least some of the areas adapted to comprise immobilised reagents, each field generating means generating a field proximate to the corresponding area whereby reagents entering the flow channel and being susceptible to the field generated at the area are attracted to and immobilised at the area or are rejected from the area. Alternatively, the width of the channel of the micro flow system can be extended to accommodate a two-dimensional grid of areas to comprise immobilised reagents with fields generating means positioned proximate to at least some of these areas. In another embodiment the micro flow system for analysing a sample with a large number of reagents simultaneously may consist of an array comprising a number of parallel channels each with a plurality of areas adapted to comprise immobilised reagents located in the flow channels and further comprising field generating means to generate a field proximate to the areas whereby reagents being susceptible to the field are immobilised at the area. The field generating means may be e.g. permanent magnets, electrodes or electromagnets.

The devices with assay sites enable rapid manipulation, detection, and analysis of macromolecules, particles and cells in biologic or chemical samples in that a plurality of tests can be performed on the same microchip. According to the invention, micro flow systems and molecular biology are combined.

BRIEF DESCRIPTION OF THE DRAWINGS

Exemplary embodiments of the invention will now be described with reference to the accompanying drawings in which

FIG. **1** illustrates the operation of particle separation according to the present invention,

FIG. **2** shows a cross-sectional view of a separation flow channel according to the present invention. (a) shows the main embodiment and (b) shows a cross-sectional view of a separation flow channel for gravitational separation,

FIG. **3** shows a micro flow system with electrodes as field generating means,

FIG. **4** shows a flow diagram of a magnetic particle separation apparatus according to the present invention,

FIG. **5** shows flow diagrams of various embodiments of the present invention. (a)–(d) show embodiments with various numbers of inlets and outlets, and (e) shows an embodiment with an enlarged separation chamber, and (f) shows an embodiment with an enlarged chamber for collecting separated particles,

FIG. **6** illustrates entrapment of magnetic particles in a flow channel,

FIG. **7** shows a flow diagram for optical detection and hydrodynamic separation using a blocking valve,

FIG. **8** shows a flow diagram for optical detection and hydrodynamic separation using syringe pumps,

FIG. **9** shows a flow diagram of two flow channels coupled in parallel (a) and in sequence (b) and (c),

FIG. **10** illustrates the principle of introducing a pretreatment facility in the member comprising the micro flow system, here further combined with a post-treatment facility or a hydrodynamic separation facility,

FIG. **11** shows a flow channel for magnetophoresis,

FIG. **12** shows a flow channel having a serial array of assay sites equipped with electrodes to immobilise probes,

FIG. **13** shows a flow channel having a serial array of assay sites equipped with magnets to immobilise probes,

FIGS. **14**(*a*) and (*b*) shows a flow channel having a two-dimensional array of assay sites equipped with magnets to immobilise probes,

FIGS. **15**(*a*) and (*b*) shows two devices each comprising a parallel array of micro flow channels each of which contains an assay site,

FIG. **16** illustrates the preparation of a micro flow system,

FIGS. **17**(*a*), (*b*), (*c*) shows diagrams from the magnetic separation described in Example 3, and

FIG. **18** is a flow chart illustrating a process for separating fetal cells from a maternal blood sample by combining different separation methods as described in Example 4.

DETAILED DESCRIPTION OF THE DRAWINGS

According to a preferred embodiment of the invention, magnetically stained particles, e.g. cells labelled immunologically with magnetic particles, such as antibody-coupled magnetic beads, are separated from non-magnetic particles, i.e. non-labelled cells, by exposing the particles to a magnetic field generated with a permanent or an electromagnet. Positive or negative selection methods may be employed. By positive cell separation, cells of a specific cell type are separated and isolated from a heterogeneous mixture of cells.

FIG. **1** illustrates the principle of the separation method according to the invention. A micro flow system **1** is shown having three inlet and two outlet ports. The sample **9** containing particles enters the separation flow channel **5** through a central inlet port **2** and is continuously guided through the separation flow channel **5** of the micro flow

system **1** by two guiding buffers **10** and **11**, each of which enters the separation flow channel through inlet ports **3** and **4**, respectively. A field generating means comprising a magnet **8** is located adjacent to the flow channel **5** and generates a magnetic field across the flow channel **5**. When the sample **9** containing particles passes the magnetic field, magnetically stained particles **12** are drawn into the guiding buffer **10** and leave the flow channel **5** together with the guiding buffer **10** through the sort outlet **6** while non-labelled cells **13** which are not influenced by the magnetic force remain in the fluid **9** leaving the flow channel **5** through the waste outlet **7**.

Due to the small channel dimensions, the flow is laminar with negligible influence of inertial forces. Mixing of the sample flow and the guiding buffers is not detectable since the contact area is small and the contact time is reduced to a subsecond range. The thickness of the sample flow can be precisely adjusted by variation of the flow rate of the two guiding buffers. This enables the adjustment and optimisation of the magnetic micro flow system for various cell types and sizes. The volume flow of the sample and the two guiding buffers are adjusted so that the particles in the sample are lined up into a single stream of particles.

The magnetic field in the micro flow channel operates as an extremely sensitive filter for magnetic particles, e.g. cells. Cells labelled with superparamagnetic beads (e.g. MACS, Dynal) are magnetised and attracted by the magnetic field whereby the flow of magnetised particles is deflected into the sort outlet. The short residence time of the fluids in the flow channel and the low Reynolds numbers of the flow in the flow channel minimise the influence of gravity compared to the influence of the magnetic force.

FIG. **2** shows a cross-sectional view of two variants of the micro flow system **1** manufactured utilising semiconductor technology. The micro flow system may be manufactured in any suitable material such as polymers, glass, semiconductors, such as silicium, germanium, gallium arsenate, etc., etc.

The first micro flow system (a) shown is a 3-layer sandwich. The central layer **14** is a silicon wafer having a flow channel **5** etched into it. The silicon wafer **14** is covered with a transparent plate **15**, such as a glass plate, having a thickness of, e.g., 0.16 mm. Fluids inside the flow channel **5** may be observed through the glass plate **15**, e.g. utilising a microscope **16** (detection means). The fluid inlet **2** and outlet **7** are connected to tubings **17**, **18**, e.g. fused silica capillary or Teflon tubings, for entering fluids into or discharging fluids from the flow channel **5**. Buffer inlets **3** and **4** and the outlet **6** for the separated particles are not shown. The bottom plate **19**, e.g. made of plastic, facilitates mounting of the tubings **17**, **18**.

A modified version (b) of the micro channel system for separation was designed with gravitation as the force field, thus sorting particles due to their density and/or diffusion constant, the latter mainly being controlled by the shape and size of the particles. The system is during operation positioned with the flow plane substantially perpendicular to the direction of the force of gravity. As illustrated in FIG. **2**(*b*), this embodiment of a micro flow system **1** has a sample inlet port **2** and an outlet port **7** located above the micro channel **5** and a buffer inlet port **3** and an outlet port **6** located below the micro channel **5**. The sample containing particles **9** enters the separation flow channel through inlet port **2**, and a guiding buffer **10** enters the separation flow channel **5** through inlet port **3**. In this way, two laminated layers of fluid extending along the horizontal plane are created continuously flowing through the separation flow channel **5** of the micro flow system **1**. Particles move from the particle containing layer to the guiding buffer layer by sedimentation. When the sample containing particles **9** passes the flow channel **5**, particles with certain density and size properties are drawn into the guiding buffer **10** by the gravitational force and leave the flow channel **5** together with the guiding buffer **10** through the outlet port **6** while particles which are less susceptible to the gravitational field remain in the sample **9** leaving the flow channel **5** through the waste outlet **7**. The vertical displacement of a specific particle in the sample is given by its density and diffusion constant and the contact time of the sample layer with the guiding buffer layer. The contact time is defined by the total flow rate of the fluids passing through the micro systems **1** and the length of the micro channel **5**. The system can be adjusted such that a desirable or appropriate specimen can be withdrawn and separated from the sample flow due to their density and/or diffusion properties by adjusting the volumetric flow rates of the guiding buffer and particle containing sample.

Alternatively, the micro flow system may comprise two further inlet ports for entering a second and a third guiding buffer into the micro channel **5**, where the two further inlet ports are positioned above the micro channel, one on each side of the sample inlet port **2**. The flow rates of the sample and the second and third guiding buffers may be adjusted so that the particles contained in the sample are lined up in a single line.

Characteristic features of an exemplary embodiment of a micro flow system according to the invention, e.g. as shown in FIGS. **1** and **2**, is shown in Table 1.

TABLE 1

Characteristics, micro flow system	
Manufacturing method	
Material: Silicium	
Oxide, SiO_2	
Photo-lithography	
Wet-chemical etching	
Flow Channel	
Cross sectional area	0.1–0.55 mm width × 0.04–0.2 mm depth
Length	1.0–200 mm
Total flow rate [μl/min]	1–200
Flow velocity [mm/min]	15–180
Reynolds number	0.1–20
Separation time	0.1 sec–6.0 sec [2 μl/min]
Magnet	
Permanent Magnet	
Rare Earth Samarium-Germanium	0.5 × 0.5 × 0.2 mm
Electromagnet	
Holding Magnet	25 mm 12 V D.C. RS

FIG. **3** shows a micro flow system **1** utilising electrodes **20**, **21** to generate an electric field across the flow channel **5**. The electrodes **20**, **21** may introduce dielectrophoretic or electrophoretic forces utilised for particle separation. For electrophoretic separation to take place, gold electrodes may be positioned at the inside of the walls of the flow channel **5**. By applying a voltage across the electrodes, an electrical field is generated substantially perpendicular to a longitudinal axis of the flow channel. The electrical field cause deflection of charged particles or molecules in the flow channel **5** whereby electrically charged particles can be deflected away from the sample containing particles flowing in the micro flow channel and into a guiding buffer also

flowing in the flow channel and abutting the sample containing particles in the micro flow channel.

FIG. **4** shows a micro flow apparatus **22** including a micro flow system **1** as shown in FIGS. **1** and **2**. The micro flow system **1** has two inlets **2, 3** and two outlets **6,7**, two syringe pumps **23, 24**, two 3-way control valves **25,26** and capillary tubings **27, 28**. The capillary tubings **27, 28** are used for interconnecting the two syringe pumps **23, 24** with the inlets **3, 2**, respectively, of the micro flow system **1**.

Conventional syringe pumps with means, e.g. stepping-motors (not shown), to move the pistons at a predetermined speed have been utilised for generating a continuous flow of the guiding buffer through the inlet tube **27** and a continuous flow of the sample through the inlet tube **28**. The system can be operated in a first loading mode where the two 3-way control valves **25, 26** open for flow between the syringe pumps **23, 24** and the buffer reservoir **29** and the sample reservoir **30**, respectively, and the syringe pumps **23, 24** are loaded with buffer and sample from the reservoirs **29, 30**, respectively. In a consecutive second operational mode the two 3-way valves **25, 26** open for flow between the syringe pumps **23, 24** and the capillary tubing **27** to the buffer inlet **3** and the capillary tubing **28** to the sample inlet **2** of the micro flow system **1**, respectively. The syringe pumps are in this second operational mode controlled to generate a predetermined volumetric flow rate through the micro flow system **1**.

FIG. **5** illustrates various micro flow systems **31, 32, 33, 34, 35**, and **36** having flow channels of different geometries, illustrating different embodiments of the invention. Micro flow systems with two or three inlet ports and two, three or five outlet ports, respectively, are shown in FIGS. **5**(*a*)–(*d*). The system shown in **5**(*a*) with inlet ports for sample and two guiding buffers, respectively, and sort outlet port and waste outlet port is similar to the system shown in FIG. **1**. FIGS. **5**(*b*) and (*c*) show systems with multiple outlet ports, three and five, respectively, whereto particles can be sorted and leave the flow channel through according to their susceptibility to the applied field. A simple system with two inlet and two outlet ports are shown in FIG. **5**(*d*) similar to the one in FIG. **2**(*b*) that is used for gravitational sorting. A micro flow system with a separation channel equipped with a magnet where the width of the separation channel is enlarged before the bifurcation in a sort outlet and a waste outlet is shown in FIG. **5**(*e*). According to the behaviour of liquids in a flow channel, the size of the cross-sectional area occupied by the sample flow is proportional to the width of the separation channel. According to this, the transversal distance between two particles A and B is increased proportional to the increase of the width of the separation channel. A larger distance between particles, which are to be separated, yields a higher selectivity of the mechanical separation. FIG. **5**(*f*) shows a micro flow system where the width of the outlet channel **6** is increased to form a chamber where the sorted particles are collected for further processing or analysis, e.g. detection, staining, destaining or cultivation.

FIG. **6** illustrates a system in which particles are entrapped inside the micro flow channel **5** for a desired period using the electromagnet-equipped apparatus. In this case, the magnetic field is adjusted so that magnetic particles **12** are drawn to the inner wall of the micro flow channel **5** close to the electromagnet **8**. Upon removal of the current to the electromagnet **8** the particles **12** are redispersed and are rapidly moved to the sorting outlet port **6**. This 2-step sorting procedure is an alternative to the continuous sorting procedure that is particularly useful in sorting of extremely rare events where dilution of the sorted cell fraction could be a problem. The sorting outlet port **6** may be closed when the current to the electromagnet **8** is turned on and is open when the current to the electromagnet **8** is turned off. The figure shows magnetic particles **12** in the process of being withdrawn from a continuous sample flow **9**. The magnetic particles **12** are attracted by the magnetic field and withdrawn from the sample flow **9** by precipitation at the inner wall of the micro flow channel **5** proximate to the electromagnet **8**. When the current supplied to the electromagnet **8** is turned off, the magnetic particles **12** are released into the flow again. The separation flow channel may not have a sort outlet, instead a buffer may enter the micro flow channel **5** after the sample and the entrapped particles may be released by removing the current supplied to the electromagnet **8**.

FIG. **7** illustrates another embodiment of the present invention for separation of particles fulfilling certain criteria from the sample **9** by hydrodynamic force. The apparatus comprises a 2-way valve **40** and a micro flow system having a separation flow channel **5** with three inlets **2, 3, 4**, two outlets **6, 7** and a collecting chamber **37**. The sample **9** containing particles enters the separation flow channel **5** through a central inlet port **2** and is continuously guided through the separation flow channel **5** of the micro flow system by two guiding buffers **10** and **11**, each of which enters the separation flow channel **5** through inlet ports **3** and **4**, correspondingly. The sample **9** is monitored utilising a microscope objective **16**. The apparatus has control means **38** for controlling the two-way valve **40**. The control means comprises monitoring means having an optical detection means, e.g. a photomultiplier system (PMT), a CCD camera/chip or a photo diode, optically connected to the microscope objective **16**. The objective **16** is focused on the measuring volume, which is located inside the flow channel **5**. The size of the measuring volume is defined by a pinhole or slit **39** positioned in front of the optical detector and by the magnification of the objective **16**. The 2-way valve **40**, e.g. a piezoelectric drop-on-demand ink-jet printing valve, is connecting the collecting chamber **37** to the sort outlet **6**. The flow restriction of the waste outlet channel **7** is much higher than the flow restriction of the sort outlet channel. This can be achieved by attaching a flow restrictor (not shown) to the waste outlet channel **7**. Thus, if the 2-way valve **40** is open the sample **9** containing particles is deflected towards the sort outlet **6**. The collecting chamber **37** is used to collect and capture the sorted particles for post-analysis. Other particles continue to flow out through the waste outlet **7**.

Particles are physically separated using hydrodynamic forces according to optical measurements on each particle. The photomultiplier (PMT) signal generated when a particle resides in the measuring volume is transmitted to a pulse-height analyser also comprised within the control means **38**. A selection circuit provides an activating signal whenever a specific particle exhibits photometric properties of a predetermined type. If the PMT signal for a specific particle indicates that the particle is of a specific type an actuation pulse is produced. The valve **40** opens at the actuation pulse, causing the liquid containing the specific particle to flow through the sort outlet **6** and to be captured inside the collecting chamber **37**. The duration of the actuation pulse is made sufficiently long for the desired particle to be transported into the collecting chamber **37**. For light excitation several sources can be used, e.g. laser, tungsten lamp, photo diode. For bundling of the light, a fibre optic cable, a photo lens, an objective or a light microscope can be used. Various optical detection methods, e.g. fluorescence, absorbency, can be used.

The micro flow system may be positioned on a movable table so that the micro flow system may be moved into selected positions relative to the microscope whereby an appropriate volume of the micro flow channel may be moved into the measurement volume of the apparatus.

During or after sorting, the captured sample can be analysed again, using e.g. a microscope. When the valve **40** is closed, particles are entrapped inside the collecting chamber **37** and can be observed for a desired period. A desired liquid or reagent for washing, cultivation or staining of particles or cells may be entered into the collecting chamber **37**. After the separation process, the particles may be withdrawn by flushing the collecting chamber **37** with an appropriate buffer entering the micro flow system through one of the inlets **2, 3, 4**.

The sorting apparatus was designed to achieve a minimal dilution of the separated sample fraction. Hydrodynamic separation of particles can be performed due to the optical, electrical, magnetic and/or other properties of the particle-containing sample.

An example of an optical and mechanical arrangement of the apparatus based on fluorescence detection is illustrated schematically in FIG. **7**. The sample **9**, e.g. particle suspension, is guided through the separation channel **5** by two guiding buffers **10, 11** so that the particles contained in the sample **9** are lined up in a single stream flowing in a plane perpendicular to the optical axis of the objective **16**. The flow is illuminated with a mercury arc lamp passing excitation filters for e.g. fluorescein measurement. A dicroic mirror reflects the excitation light to the sorting chip via e.g. a 20x microscope objective **16**. The fluorescence light emission is collected by the same objective **16** passing a dicroic mirror. Behind the mirror, a slit **39** works as field stop limiting the detection area to a small stripe. Each particle passing the objective **16** is generating a short signal from the photomultiplier that is optically connected to the objective **16**. The photomultiplier signal is amplified and transmitted to a peak detector.

The actuation frequency of the valve **40** used in this device is 1500 Hz which corresponds to a minimal actuation time of 0.6 msec.

FIG. **8** illustrates an alternative embodiment of the separation apparatus shown in FIG. **7** with a separation flow channel **5** having three inlets **2, 3, 4** and two outlets **6, 7**. The sample containing particles enters the separation flow channel **5** through the centre inlet port **2** and two guiding buffers enters the channel **5** through the other two inlet ports **3, 4**, respectively. Flow speed adjustment means comprising stepper motor driven syringe pumps **41, 42** are connected to the two outlet ports **6, 7**, respectively. The syringe pumps **41, 42** suck the sample and buffer via inlet **2, 3** and **4**, respectively, through the separation flow channel **5**. The cells are monitored at the optical axis of the microscope objective **16** and flow to the separation junction. The guiding buffers and the sample containing unselected cells flow out into the waste outlet syringe pump **42**. If a specific cell has optical properties causing an actuation pulse, the stepper motor of the pump **41** at the sort outlet is actuated and the stepper motor of the pump **42** at the waste outlet is stopped causing the liquids to flow to the sort outlet **6**. The period the pump **41** at the sort outlet is switched on, respectively the pump **42** at the waste outlet is switched off is made sufficiently long to ensure that the desired cell has entered into the collecting chamber **37**. When one of or both syringe pumps **41, 42** after some operation time need to be emptied, the 3-way valves **43, 44** are switched from their normal operation position where they open for flow from the separation flow channel **5** to the syringe pumps **41, 42**, respectively, into a position where the 3-way valves **43, 44** open for flow between the syringe pumps **41, 42** and a waste container (not shown) and where the stepper motors driving the syringe pumps **41, 42** are operated in the reverse direction of the normal operation direction to empty the syringe pumps **41, 42**.

FIG. **9**(*a*) shows two flow channels **45, 46** operating in parallel. The sample containing particles enters the flow channels **45, 46** through inlet ports **47, 48**, respectively. The guiding buffer enters the flow channels through the inlet ports **49, 50**, respectively. In the flow channels **45, 46**, particles susceptible to the magnetic field generated by magnets **51, 52**, respectively, are deflected from the sample containing particles into the corresponding guiding buffer and flow thereafter through the sort outlet **53**. The remaining part of the sample leave the flow channels **45, 46** through the waste outlets **54, 55**, respectively. Separation is increased by using a plurality of flow channels coupled in parallel.

FIGS. **9**(*b*) and (*c*) shows examples of combinations of micro flow systems for magnetic, hydrodynamic or gravitational separation. In FIG. **9**(*b*), particles are first separated from a sample in a magnetic separation channel, where after the sorted particles are subjected to a hydrodynamic separation due to the optical properties of the particles. Thus, it is possible to analyse and separate particles from a sample based on both optical and magnetic properties of the particles or to another combination of properties or characteristics. In FIG. **9**(*c*), two magnetic separation channels are coupled in series in order to obtain a highly purified product.

FIG. **10** illustrates examples of micro flow systems having means for automated labelling of particles with fluorescence or magnetic probes. The system may be combined with post-treatment means for removal of the probes or for other treatment of the sorted particles. The system contains a micro flow system containing channels **56, 57** for addition of liquids to the sample, e.g. reagents for cell lysis or staining, a channel **58** for incubation and cultivation or storage of the sample for further processing and a separation channel **5**. A sample is introduced into the micro flow system via an inlet **2** and one or more reagents can be added continuously to the sample, which is transported into the incubation channel **58**. A simple micro flow structure was constructed for sample pre-treatment. Preferably, the flow rates are managed by computer-controlled syringe pumps. The incubation period between mixing and analysis of the sample is given by the volumetric flow rate of the syringe pumps and the cross-sectional area and length of the incubation channel.

FIG. **11** shows a micro flow system for magnetic separation of macromolecules, i.e. ribonucleic acid or proteins from a sample Magnetic beads labelled with a fluorescence dye and a probe, specific for i.e. DNA are added to the sample which is then incubated. This sample is entered via inlet port **2** into the separation chamber **5** and the particles are drawn by the field generated by the magnet **8** along the separation channel **5** due to their mobility. After a defined period, the magnetic field is removed and the fluorescence banding can be observed under a microscope. By running standards of known size, it is possible to calibrate the system and to separate particles of e.g. DNA due to their size and shape, similar to electrophoresis.

FIG. **12**(*a*) with details in FIG. **12**(*b*) shows a serial sensor array. A micro flow channel **5** has a plurality of assay sites, each equipped with field generating means **59** that may be individually turned on and off. The flow channel **5** shown has rectangular electrodes **59** positioned in small grooves at

the bottom wall of the flow channel **5**. A voltage can be applied selectively to each electrode **59**. Various probes, receptors, indicators, etc. may be attracted to and immobilised at selected electrodes **59** by applying a voltage to the selected electrodes **59** while a fluid containing the corresponding probes, receptors, indicators, etc. resides in the flow channel **5**. Preparation of the multiple assay sites may be accomplished by sequentially loading each assay site with a specific probe. Voltage is applied to one or more specific electrodes in the micro flow channel **5**, and a fluid containing a specific probe, reagent or indicator, etc. is entered into the micro flow channel **5** where the probes etc. will be attracted to the electrodes to **59** which the voltage is applied. Subsequently, the voltage is turned off. Then, a voltage is applied to the next electrode **59** and the next fluid containing a specific probe etc. is entered into the micro flow channel **5**. Thus, various assay sites each containing a specific probe, reagent, or indicator can be created. Antibodies, fluorescence molecules, DNA, RNA and protein dyes are examples of probes.

As an alternative to the electrodes **59**, magnetic force can be selectively applied to the assay sites with an array of electromagnets positioned at or near the surface of the micro flow channel **5** to immobilise probes etc. that have magnetic properties to desired assay sites. Alternatively, a photoactivation process can be used for covalent coupling of molecules or particles to the surface of the channel **5** at the assay site.

One example of a probe is DNA, which has an overall negative charge, drawn to the electrode **59** surface by a positive bias, another example is DNA-coated magnetic particles that are drawn to the surface of micro flow channel **5** by magnetic means.

By modification or coating of the surface of the micro flow **5** channel and/or the electrodes **59** or magnets, specific chemical and mechanical properties can be created. To increase the binding forces of the probe, the surface may be coated with a specific layer or matrix, e.g. a polymer such as urethane or a reactive chemical group. When the current to the selected electrode or electromagnet is switched off the probe remains on the surface e.g. by absorption. Thus, an encapsulation or immobilization of the molecule is achieved.

Thus, a field generated at selected assay sites across the flow channel **5** may be utilized for immobilization of particles, such as biomolecules, whereby these may be held in substantially fixed positions within the flow channel permanently or for a specific period of time allowing chemical reactions between the particles and an entered reagent to take place and/or kinetic measurements on the particles to be performed and/or the particles to be brought into contact with different chemical substances. For analysis of the reactions in the micro flow system, optical detection means, e.g. a microscope, may be used.

It is an important advantage of the device that a number of assays can be performed in a single device. During operation of the device, various processing steps, such as e.g. washing steps, and reagent addition, etc., may be performed.

FIG. **13** shows a micro flow device with a flow channel **5** and with a serial array of assay sites and permanent magnets **8** positioned on a separate cartridge **60**. A second cartridge **61** has a flow channel **5** with an inlet **2** and an outlet **7**. The cartridge **60** carrying the magnets **8** can be positioned exactly below the second cartridge **61** so the magnets **8** are accurately positioned below the assay sites in the flow channel **5** as shown in the figure below cartridge **61**.

Probes to be immobilised at a specific assay site utilising a magnetic field, as described in FIG. **12** or FIG. **13**, may be positioned at the desired assay site by a method comprising the steps of positioning a defined volume of the liquid containing the magnetic probe or reagent using e.g. inkjet based dispenser technology, within a specific volume of the flow channel **5** right over one of the permanent magnets **8** for immobilization of the magnetic probe or reagent in an assay site at the surface of the flow channel **5**. The method may be repeated for various probes to be immobilised at various assay sites, respectively. After the immobilization, the cartridge **61** containing the flow channel **5** is covered by a transparent cartridge **15**, e.g. a glass plate, allowing the assay site array with the probes inside the micro flow channel **5** to be observed. An analysis with the assay site array is performed by introducing a sample through inlet **2** into the micro flow channel **5** where it passes the array of assay sites and leaves the micro flow channel **5** through outlet **7**. An objective **16**, optically connected to an optical detector, e.g. a fluorescence microscope, may be focused on the array in the micro flow channel **5** to monitor the chemical reactions at the assay sites.

FIG. **14** shows another embodiment of the invention comprising a cartridge **62** with a micro flow channel containing assay sites **63** arranged in a two-dimensional array, a cartridge **64** with permanent magnets **8** and a transparent cartridge (not shown) to cover the cartridge **62** with the micro flow channel. The assay sites **63** are formed as small grooves at the surface of the bottom wall of the micro flow channel. The dimensions of the cartridges **62**, **64** and the position of the assay sites **63** and the magnets **8** are the same, so if cartridge **62** is placed over cartridge **64** as shown in FIG. **14**(*b*), the magnets **8** are located under the assay sites **63**.

The embodiments shown in FIGS. **12**, **13** and **14** may be utilised for hybridisation of DNA as described below. A magnetic carrier including a DNA probe may be immobilised at a specific assay site as described previously. In this way, an array of assay sites is created in a micro flow channel wherein each assay site contains a different DNA probe. Thereafter, a sample containing target molecules is entered into the micro flow channel preferably until the sample has filled the micro flow channel. After the target molecule has been hybridised to a DNA of a specific assay site, a solution of reporter probes, e.g. fluorescence probes, is entered into the flow channel where it binds on the assay site carrying the hybridised DNA. By using a fluorescence detector, e.g. a photomultiplier, focused on the different assay sites the reaction at each assay site can be monitored. By removing the magnetic field on a specific assay site the magnetic material including the DNA probe can be removed, so the process can be reversed. Thus a renewable array of assay sites can be created and wash processes can be implemented in the operation of the array of assay sites.

FIG. **15**(*a*) shows a device according to the invention for performing a multiple assay analysis in a micro flow system by using a parallel array of assay sites. The system comprises an array of parallel micro flow channels **65** each of which contains one assay site with a specific probe immobilised using e.g. an electrical or magnetic field or by photoactivation as described previously. For example, a cartridge containing a permanent magnet (not shown) can be positioned below the parallel micro flow channels **65** in whereby magnetic probes can be immobilized in the micro flow system using dispensing technology. In this way, a plurality of assay sites may be created in the parallel flow channel **65** array allowing a simultaneous analysis of a

sample with a plurality of probes or reagents defined by the number of parallel micro flow channels **65**.

The micro flow system consists of two parallel flow channels **66**, **67** which are connected via a number of parallel micro flow channels **65** each containing an assay site. An injection flow channel **66** has an inlet **2** and is connected to an outlet **68** via a blocking valve **69**, and the waste flow channel **67** is connected to a waste outlet **70** via a blocking valve **71**. By blocking one of the two outlets **68**, **70** with the blocking valves **69**, **71**, respectively, it is possible to guide the injected flow through the array of channels **65** containing assay sites or through the injection channel outlet **68**, respectively. The flow through all channels **65** containing assay sites is merged into the waste channel **67** and is leaving the system via the waste outlet **70**. During passage of the channels **65** containing the assay sites, the sample comes into contact with sensing probes, which are immobilized at the assay sites. Chemical reactions may be detected as described for FIG. **12**.

In FIG. **15**(*b*) an alternative embodiment of an array of parallel channels **65** containing assay sites is shown. The micro flow system has three inlet ports **2**, **3**, **4** to enter different liquids into the micro flow system. By connecting outlet port **68** to a flow restrictor, only one blocking valve **71** is needed to operate the system. If the blocking valve **71** between the channel array **65** and the waste outlet **70** is closed, the channel array **65** is blocked and the flow from inlet **2**, **3**, **4** will pass the injection channel **66** and leave the micro flow system via outlet **68**. When the blocking valve **71** is open, the liquid introduced in the injection channel **66** will flow into the sensor channel array **65** because of the higher flow restriction at outlet **68** compared to waste outlet **70**.

FIG. **16** shows a micro flow system manufactured as a 3-layer sandwich. The central layer is a silicon wafer having a flow channel etched into it. The silicon wafer is covered with a transparent plate, such as a glass plate, having a thickness of, e.g., 0.16 mm. Fluids inside the flow channel may be monitored through the glass plate, e.g. utilising a microscope or other optical detection means. The fluid inlet and outlet are connected to tubings, e.g. fused silica capillary or Teflon tubings, for entering fluids into or discharging fluids from the flow channel. Buffer inlets and the outlet for the sorted particles are not shown. The bottom plate, e.g. made of plastic, facilitates mounting of the tubings.

FIGS. **16**(**1**) to (**5**) illustrates the following description of the manufacturing and preparation of a micro flow system. A separation flow channel was designed to fit into a system comprising a bonded silicon/glass sandwich. The micro channels were etched into a silicon wafer covered with a boron glass plate having a thickness of 0.2 mm allowing monitoring of the micro channels, using i.e. a microscope. The separation flow channel was fabricated on a 4", 350 μm, <100> silicon wafer. A 1.5 μm layer of SiO_2 was applied to the surface of the silicon wafer and was patterned with a mask containing the channel layout. A 2.6 μm layer of photoresist was spun on top of the SiO_2 and patterned with a mask defining intermediate holes. The two-step mask consisting of a SiO_2 mask and a photoresist mask was used for etching a two level structure with vertical walls by reactive ion etching (RIE) in a SF_6:O_2 plasma. The holes were initially etched to a depth of 22 μm and then etched deeper together with the channels, which were etched to depths in the range from 40 μm to 100 μm. A layer of 1.8 μm SiO_2 was patterned with a mask for inlets and outlets on the back of the silicon wafer. The etching was carried out in KOH at 80° C. and was stopped when all the intermediate holes were clearly visible from the back. Finally, a glass wafer was anodically bonded to the silicon wafer. The micro channels were designed for volumetric flow rates of 0.1 to 200 μl/min with a mean flow speed of maximum 100 (mm/min).

The separation flow channel may be provided with one or two permanent or electromagnets in three different ways:

(a) Rare earth Samarium-Cobalt block magnets of 1.0×1.0×0.5 mm (Goudsmit, Netherlands) may be glued with silicon rubber into the opening slot of the separation flow channel.

(b) Rare earth (Sr) magnetic powder (Iropag, Hamburg, Germany) can be mixed with epoxy 1:1 (v/v) and this magnetic paste may be glued into the opening slot of the separation flow channel yielding a thick film magnetic layer of 1.0×1.0×0.5 mm.

(c) Ferrite steel wool may be glued with silicon rubber into the opening slot of the separation flow channel. A high magnetic field gradient can then be induced inside the opening slots by applying an external magnetic field, e.g. by an electromagnet (Goudsmit, Netherlands) positioned proximate to the separation flow channel.

EXAMPLE 1

A micro flow system with a layout as sketched in FIG. **5**(*d*) with two inlets and two outlets has been tested utilising it for separation of various magnetisable particles. The test conditions are listed below.

Particle concentration	10^7 particles/ml
Total flow rate	25 μl/min
Length flow chip	3.5 mm
Channel width	250 μm
Channel depth	60 μm
Separation time	2.4 sec
Desired particle deflection:	10 μm

The separation efficiency (enrichment rate) E and depletion rate 1/E are defined by

$$E = \frac{\%\ positive\ particles\ after\ separation}{\%\ negative\ particles\ after\ separation} \Big/ \frac{\%\ positive\ particles\ before\ separation}{\%\ negative\ particles\ before\ separation}$$

For separation of various paramagnetic standard beads of different sizes and paramagnetic field strength, the results are shown in the Table 2.

TABLE 2

Separation efficiencies

Paramagnetic Bead	Size μm	Separation Efficiency [%][1] A)	B)	C)
Polysciences				
25% iron-oxide	1–10	>99	>99	95
50% iron-oxide	1–10	>99	>99	96.5
Paesel + Lorei				
Magnetic Affinity	0.5–1.5	>99	>99	97.5

TABLE 2-continued

Separation efficiencies				
		Separation Efficiency [%][1]		
Paramagnetic Bead	Size μm	A)	B)	C)
Boehringer				
Streptavidin Magnetic Dynal	1	90.5	88.7	89.5
Magnetic Mass Dyal M-450	1–10	98.0	>99	96.5

[1]total flow rates:
A) = 10 μl/min,
B) = 50 μl/min.
C) = 100 μl/min

EXAMPLE 2

Further, the micro flow system used in Example 1 has also been tested by utilising it for separation of Human T-lymphocytes (JURKAT cells). Magnetically stained and unstained JURKAT cells were used to form a heterogeneous cell sample. For magnetic staining of the cells, a CD4-magnetic surface marker from Miltenyi Biotech was used. JURKAT cells were suspended in 1% PBSIBSA to a concentration of 10^7/ml. Biotin-conjugated CD4 magnetic microbeads were added at 2 μl stock/10^7 cells following the manufacturer instruction.

The magnetically stained cells (10^7 cells/ml) flowed through the microchip for 10 min. and fluids were collected at the two outlets. Three experiments at different flow rates (5, 25, 50 μl/min) were performed. The same experiments were performed using magnetically unstained cells.

An aliquot of the collected samples was analysed under a microscope and the particles were counted using a Neubauer microscopy chamber. For each experiment 1 μl sample was analysed:

Run	flow rate [μl/min]	cells [%] at Sort outlet
Negative (unstained cells)	5	<0.1
	25	<0.1
	50	<0.1
Control[1]	5	n.n.
	25	n.n.
	50	n.n.
Positive (stained cells)	5	95.5
	25	92.8
	50	80.5
Control[1]	5	n.n.
	25	n.n.
	50	n.n.

[1]using the micro flow system without an integrated magnet

EXAMPLE 3

The system employed for separation of magnetisable particles from a sample is shown in FIG. **4**. It comprises two syringe infusion pumps (Harvard Apparatus, Southnatik, Ariz.) that provides constant flow rates of 0.1 to 100 μl/min using a 0.5 ml micro syringe (Hamilton, Bonaduz, Switzerland), a separation flow channel of silicon for the separation of the magnetisable particles, and a collecting unit for collecting of the sorted particles. Two 3-way microvalves (Lee, Parameter AB, Sweden) were integrated into the apparatus for sterile solution handling. All components were interconnected with fused silica capillaries (340 μm id., Supelco, U.S.A.). The SFC was place under an inverted microscope (Axiovert 100, Zeiss, Germany) for visualisation of the seperation procedure. All micro channels and tubing were deactivated by silanisation as described in Blankenstein, G. Scampavia L, Branebjerg J, Larsen U D, Ruzicka J (1996): Flow switch for analyte injection and cell/particle sorting in Analytical Methods and Instrumentation, μTAS '96 conference, Nov. 17–22 1996, Basel. A FACScan with 488 nm argon laser excitation and collection of forward and side scatter and fluorescence of fluorescein were used (Becton Dickinson, Mountain View, Calif.) for all experiments. Results were collected and analysed using the FACScan research software (Becton Dickinson).

Results on the use of a separation flow channel equipped with a permanent magnet optimised for Dynal beads are shown in FIG. **17**. A bead suspension of 1–5x10^8 particles/ml containing a mixture of non labelled magnetic Dynal particles (d: 4.5 μm, M-450) and fluorescence calibration beads (d: 3.2 μm, Dako AIS, Glostrup, Denmark) have been separated. About 1 ml of the non-magnetic, non-deflected fraction was collected at the waste outlet and analysed by flow cytometry (B). To enumerate the positive and negative fractions, two windows were set for the statistic evaluation. Before separation, the sample contained 38.3% fluorescence particles and 55.8% magnetic particles, respectively (a). After sorting by the described system almost all magnetic particles were found in the sorted fraction collected from the sort outlet (b) and non-magnetic particles were found in the negative fraction (c) collected from the waste outlet, respectively. Under optimised conditions, an enrichment rate of 350 was achievable.

EXAMPLE 4

This example concerns enrichment of fetal cells in a sample for magnetic activated cell sorting. A combination of the embodiment of the invention as shown in FIGS. **7** and **10** (upper), optical cytometry, and FIGS. **4** and **10** (lower), magnetic cell separation, provides a powerful apparatus for efficient enrichment of fetal cells in a sample.

The process for increasing the concentration of fetal cell in maternal blood samples involves the following steps (see FIG. **18**): (i) A first selection step for removal of the majority of the maternal blood cells based upon their volume, size and density; (ii) A second sorting step for isolation of the fetal blood cells from the remaining maternal blood cells based on immuno-fluorescent separation using a device as described in FIG. **7** and/or based on immuno-magnetic separation using a device as described in FIG. **4**. In the examples shown in FIG. **9**(*b*), the magnetic blood sample is first separated in a magnetic separation chamber, followed by a separation due to optical properties of the sample, or two magnetic separations are performed one after the other, see FIG. **9**(*c*), in order to obtain a highly purified product.

An example of sorting of particles of very low concentration from a sample of maternal blood in a non-invasive prenatal screening test is presented in the following paragraph.

Nucleated red blood cells are found in maternal blood in a concentration of 10 to 1000 per ml of all nucleated cells. Bianchi has shown (D. W. Bianchi, Journal of Pediatrics, 1995, 127, 6, p. 847–856) that it is possible to use such cells for genetic screening in prenatal diagnosis. The cell surface

marker CD71+ for example, is an appropriate marker to select such cells from maternal blood. Test results demonstrates that magnetic activated cell sorting is powerful enrichment system for sorting and isolating fetal nucleated blood cells from maternal blood. For this the magnetic activated cell micro technology as described in this invention is used. Fetal cells are distinguished and separated from maternal blood by the use of a specific surface marker (CD71) which is only present on the cell membrane of fetal nucleated blood cells. By selectively attaching a magnetic antibody probe to CD71, a magnetic probe is attached substantially exclusively to fetal cells.

EXAMPLE 5

This example concerns depletion of magnetically labelled CD45 positive cells (maternal leukocytes) from a maternal blood sample spiked with cord blood. A flow chip described in FIG. **1** was used in a system as described in FIG. **4**. In this experiment a 1:3 spike (fetal/maternal, v/v) was used to demonstrate the performance of the magnetic separation. Heparin was used as an anti-coagulant. The nucleated cells were labelled with CD45 coated magnetic 0.1μ micro particles (Immunicom, U.S.A.), using a monoclonal antibody against CD45 as the first layer. The cell suspension was collected at both outlets **6** and **7** (see FIG. **1**). For testing the sorting efficiency, parts of both the collected fractions were analysed on microscope slides. The results showed that most of the cells, more than 95%, collected at the sort outlet **6** were CD45 positive.

EXAMPLE 6

Fluorescence activated cell sorting using the device described in FIG. **7**. First results have shown an enrichment factor of more than 300, which indicates that the employed device is a powerful tool for enrichment of rare cellular events.

EXAMPLE 7

An example is given for the embodiment of the invention as described in FIG. **12** for the use of multiple sensor array technology for sensing of a group of analytes in one step. For this purpose, the biosensing components such as antigens or antibodies can be loaded into a specific assay of the flow channel and immobilised there.

Magnetic particles carrying an antigen probe are immobilised on the surface of the micro flow channel by magnetic means. The immobilisation of each probe is exactly specified to a site by switching on a specific electromagnet.

After loading the surface with different groups of antigen probes, the test solution is guided through the flow channel **5** of the microchip. If the sample contains an antibody, which is complementary to one of the different antibodies, it will bind to that specific site where this antibody is immobilised. In a third step, the sample solution has to be removed, and a liquid containing a secondary antibody against the FC region of the first antibody is guided through the micro flow channel. The secondary antibody is coupled to a fluorescence dye allowing the identification of a specific assay site whereto the antibodies has been binded. The device can be used for rapid screening of blood samples, e.g. for identification of bacteria or virus in blood samples having a micro flow channel with virus/bacteria specific antigen probes.

What is claimed is:

1. A micro flow system for separating particles, comprising a member having

a flow channel defined therein for guiding a flow of a fluid containing the particles through the flow channel, the flow channel having an inlet end and an outlet end, the particles remaining in the flow channel for a time, and the particles flowing in a path through the channel at a fluid flow rate, the path of flow having a width and a position,

first inlet means positioned at the inlet end of the flow channel for entering the fluid into the flow channel,

second inlet means for entering a first guiding buffer for controlling cross-section and flow path through the flow channel of the flow of the fluid containing particles, the first guiding buffer having a flow rate,

first outlet means positioned at the outlet of the flow channel for discharging the fluid from the flow channel,

the flow of the fluid containing the particles being controlled in such a way that one particle at a time passes a cross-section of the flow channel,

the member being positioned in a field that is substantially perpendicular to a longitudinal axis of the flow channel so that particles residing in the flow channel and being susceptible to the field across the flow channel are deflected into the first guiding buffer toward the field.

2. A micro flow system according to claim **1**, further comprising third inlet means for entering a second guiding buffer for further controlling the cross-section and the path through the flow channel of the flow of the fluid containing particles, wherein the first and second guiding buffer surround the flow of the fluid containing particles, the first and second guiding buffers having a flow rates.

3. A micro flow system according to claim **2**, wherein the width and the position of the flow of fluid containing particles is controlled by adjusting volumetric ratio between the fluid flow rate of the flow of fluid containing particles and the flow rate of the guiding buffers.

4. A micro flow system according to claim **1**, wherein the member further comprises field generating means positioned proximate to the flow channel for generating a field substantially perpendicular to the longitudinal axis of the flow channel.

5. A micro flow system according to claim **1**, further comprising monitoring means positioned at the flow channel for monitoring parameters of a particle residing within a measurement volume within the flow channel and providing an output signal corresponding to a monitored parameter.

6. A micro flow system according to claim **5**, wherein the monitoring means comprise optical detection means for monitoring optical parameters of a particle residing within a measurement volume within the flow channel and providing an output signal corresponding to an optical parameter.

7. A micro flow system according to claim **5**, wherein the monitoring means comprises a Hall sensor for measurement of a magnetic parameter of a magnetic particle within a specific volume of the flow channel.

8. A micro flow system according to any of claim **5**, further comprising field generating control means for controlling the strength of the field generated by a field generating means in response to the output signal of the monitoring means whereby particles are separated according to values of a parameter monitored by the monitoring means.

9. A micro flow system according to claim **1**, wherein the flow of the fluid containing the particles through the channel has a Reynolds number between 0.01 and 500.

10. A micro flow system according to claim **9**, wherein the flow of fluid containing the particles through the channel has a Reynolds number between 0.5 and 50.

11. A micro flow system according to claim **9**, wherein the flow of fluid containing the particles through the channel has a Reynolds number between 0.1 and 25.

12. A micro flow system according to claim 1, wherein a portion of the flow channel has a lowest cross-sectional area, the lowest cross-sectional area of the flow channel is between 0.004 mm^2 and 0.11 mm^2.

13. A micro flow system according to claim 1, further comprising second outlet means for discharging particles having been deflected in the flow channel.

14. A micro flow system according to claim 4, wherein the field generating means comprises a magnet.

15. A micro flow system according to claim 14, wherein the field generating means further comprises ferrite members positioned at the flow channel for focussing of a magnetic field.

16. A micro flow system according to claim 4, wherein the field generating means comprises an electrode.

17. A micro flow system according to claim 4, wherein positions in relation to the flow channel of the field generating means are adjustable for adjustment of the strength of the field across the flow channel.

18. A micro flow system according to claim 1, further comprising flow speed adjustment means for adjustment of the time the particles reside in the flow channel.

19. A micro flow system according to claim 1, further comprising a cover for covering the flow channel.

20. A micro flow system according to claim 19, wherein the cover is a transparent or translucent cover allowing optical monitoring of the flow channel.

21. A micro flow system according to claim 13, wherein the deflected particles comprise living cells.

22. A micro flow system according to claim 13, wherein the deflected particles comprise beads, microspheres, chromosomes, organelles, biomolecules, or proteins.

23. A micro flow system according to claim 13, wherein the deflected particles have been magnetically, chromophorically, or fluorescently stained.

24. A micro flow system according to claim 4, further comprising a plurality of outlets for discharging of particles separated according to their different susceptibility to the field across the flow channel.

25. A micro flow system according to claim 1, wherein the member further comprises one or more pre-treatment facilities, one or more post-treatment facilities, or one or more pre-treatment and post-treatment facilities.

26. A micro flow system according to claim 25, wherein the pre-treatment facilities comprise incubation means for preparing or pre-reacting the fluid comprising the particles.

27. A micro flow system according to claim 25, wherein the pre-treatment facilities comprise means for magnetic, fluorescent, or chromophoric staining.

28. A micro flow system according to claim 25, wherein the post-treatment facilities comprise means for collecting or concentrating the deflected particles.

29. A micro flow system according to claim 25, wherein the post-treatment facilities comprise means for bringing the deflected particles into contact with one or more reagent(s).

30. A micro flow system for analyzing components of a fluid, comprising

a member having a flow channel defined therein for guiding a flow of a fluid through the flow channel,

first inlet means for entering particles into the flow channel,

first outlet means for discharging of fluid from the flow channel and a plurality of assay sites located in the flow channel and comprising immobilized reagents whereby the fluid may be analyzed for a plurality of components while residing in the flow channel, and

field generating means positioned proximate to at least some of the assay sites for generation of a field proximate to the corresponding assay site whereby reagents residing in the flow channel and being susceptible to the field when generated at a selected assay site are attracted to and immobilized at the selected assay site, or, are rejected from the selected assay site.

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